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Nanostructured monolinolein miniemulsions as delivery systems: Role of the internal mesophase on cytotoxicity and cell internalization

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ABSTRACT

Recent advances in nanoparticle systems for improved drug delivery display a great potential for the administration of active molecules. Here, lipid miniemulsions with various internal nanostructures were loaded with the chemotherapeutic agent Paclitaxel. The goal is to assess the impact of internal structures on their efficiency. Previously the structure, the stability and the physico-chemical properties of those carriers were characterized. Modalities of action were addressed by the evaluation of their effects on the tumor cells viability, their cellular uptake by flow cytometry and confocal microscopy detection of fluorescently labeled nanostructured miniemulsions. Nanostructured miniemulsions showed variations in the cell internalization process likely due to differences in the internal structure. All paclitaxel-loaded emulsions were active reservoirs from which Paclitaxel could be released, however bicontinuous cubosomes showed the best efficiency. Considering the fact that these delivery systems can offer a new life to bioactive compounds previously abandoned due to a low aqueous solubility, these data may represent an important step towards the development of new clinical therapeutic strategies against cancers.

1. Introduction

Nanostructured miniemulsions are internally self-assembled emulsions of submicron size. Amphiphilic lipids self-assemble in water and form inverted mesophases with a large number of interfacial areas between hydrophilic and hydrophobic subspaces. Hence, those mesophases are able to be used to solubilize active compounds of various hydrophobicities. Different kind of mesophase can be produced: continuous cubic networks (Gustafsson et al., 1996), hexagonal phase (H_2) of water nano-channels (Gustafsson et al., 1997), inverse micelles organized in a cubic arrangement (Yaghmur et al., 2006), or microemulsions L_2 (Pilman et al., 1980; Yaghmur et al., 2005) which are all thermodynamically stable systems. *R*-(+)-limonene is a natural terpenic oil used here to tune the type of internal structure. We can fragment those mesophases in a continuous aqueous phase at their maximum swollen state with water. These dispersions stabilized by the addition of an emulsifier are called nanostructured miniemulsions.

The main objective of this study was to establish a relationship between the internal structure of lipid-based miniemulsions and their ability for effectively delivering molecules of biological interest. Indeed, most of the reported studies aimed at the development of delivery systems derived from mixtures of many components and did not take into account about their eventual organization and the involvement in their biological features. To demonstrate an impact of the possible organization of vectors on their efficacy, we formulated nanostructured lipid miniemulsions with different types of internal phase that contain Paclitaxel as anti-cancer agent. Those vectors were able to carry amphiphilic or hydrophobic molecules which we followed the intracellular location and the biological activity.

Cubosomes were found as potential vehicles for the controlled release in drug delivery due to their large internal interfacial area (Lawrence, 1994) that maximize the availability of the loaded drug. Many studies described the active research on nanostructured miniemulsions as drug delivery systems (Akbar et al., 2017).

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Moreover it was recently shown that they were suitable for intravascular administration (Biffi et al., 2017). The toxicity of lipid nanoparticles was already tested with glycerol monoolein (GMO) and phytantriol on human embryonic kidney cells HEK293 (Hartnett et al., 2015; Muir et al., 2012). The structure forming lipid selected to build miniemulsions is an important choice since GMO was found less toxic *in vitro* than phytantriol (Hinton et al., 2014). In terms of miniemulsion types, differences in cytotoxicity were attempted to be related to the internal mesophase through a study of GMO/capric acid miniemulsions towards L929 fibroblasts (Tran et al., 2015). Authors claimed that particles with less negatively curved interfaces were found to be less toxic in accordance with hemolysis assay results.

Recently, the outer surfaces of phytantriol-based cubosomes and hexosomes were also functionalized for an active targeting to facilitate binding to specific sites of tumor cells (Zhai et al., 2015). Among mesophase forming amphiphiles, monolinolein was not tested yet.

In a first part of the study, we gathered the structural characteristics of monolinolein-based miniemulsions (size, types of internal phase) and studied their kinetic stability and fate when interacting with a biological medium. In a second part, we focused on the delivery (toxicity and internalization of miniemulsions) on human primary glioblastoma cells U-87 MG. We focused on a possible relationship between its efficacy and the internal phase.

2. Material and methods

2.1. Material

The amphiphilic lipid used for producing internal mesophases is a commercial-grade form of monolinolein named Dimodan® U/J (DU), supplied by DANISCO A/S (Braband, Denmark). It consists in 96% distilled monoglycerides, of which 62% are monolinoleate. *R*-(+)-limonene –named as the oil- was purchased from Fluka (purity >96%). Pluronic® F127 (PEO_{99}-PPO_{67}-PEO_{99}), provided by BASF, acts as the emulsifier. Chemotherapeutic agent Paclitaxel (TXL) and fluorescent probe Nile Red were purchased from Sigma-Aldrich. Molecular structures are shown in Fig. 1. All cell culture reagents, Alamar blue kit and glass coverslips were obtained from Thermo Fisher Scientific. Lab Tek Chamber coverglasses were purchased from Nunc, Dutsher S.A.

2.2. Miniemulsion preparation

Miniemulsions were prepared by mixing all the components and emulsified in water by fragmentation using ultrasonic waves (Vibracell 75115 ultrasound tip) for 8 min at 160 W in a pulse mode (1 s on/1 s off). The DU/limonene mixture forming the miniemulsion internal phase was characterized by the δ weight ratio $\delta = 100 \times DU/(DU + oil)$. *R*-(+)-limonene was used as an additive to tune the type of lipid mesophase. Thus a specific internal structure was related to the δ parameter. The sterical stabilization of the emulsion droplets was ensured by the incorporation of an emulsifier (triblock copolymer Pluronic® F127), which was previously dissolved into deionized water before mixing with the lipid/oil mixtures. We worked at an emulsifier-to-(DU + oil) weight ratio of about 8%. Different miniemulsions with 1% of dispersed phase were prepared without or with either Nile Red (0.3 mg/10 g of emulsion) or TXL (50 μM for 10 g of emulsion, added prior to sonication). When TXL was added, the sonication took place in an ice-water bath to avoid an elevation of temperature, possibly responsible for the degradation of Paclitaxel.

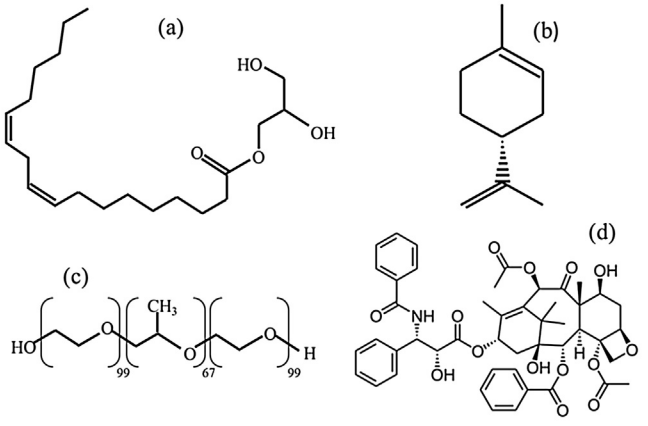

Fig. 1. Molecular structures of (a) monolinolein (18:2), (b) R-(+)-limonene, (c) Pluronic F127 and (d) Paclitaxel.

2.3. Physicochemical characterization of miniemulsions

The miniemulsion size was determined by dynamic light scattering (DLS) using a zetasizer (Zetasizer Nano ZS90, Malvern) working at λ = 633 nm and a fixed scattering angle of 90°. Samples were diluted to avoid any multiple scattering and were characterized at 25 °C. The correlation functions were automatically treated by an inverse Laplace transform giving rise to the hydrodynamic size distributions. The most representative size of the particles is taken as the mode (maximum value of the peak) derived from the size number distribution, which is of a log-normal shape. Each mode value is derived from the average of 3 measurements; each measure includes 11 runs of 10 s each. The electrical net surface charge of miniemulsion droplets was probed by zeta potential measurements using the same apparatus.

The dispersed lipid mesophase was determined by small angle X-ray scattering (SAXS). The scattered intensity was collected as a function of the wave vector $q = 4\pi\sin(\theta/2)/\lambda$ with θ the scattering angle and λ the photon wavelength. Measurements were conducted on the DELTA synchrotron BL9 beamline (Dortmund, Germany) working with a typical photon flux of 5×10^9 photons/s/mm^2 at 13 keV and with a wavelength of λ = 0.239 nm. For each sample the scattered intensity was recorded during 600 s on an image plate scanner MAR345. The measurements covered a scattering q range from 0.2 to 3.5 nm^{-1}. SAXS data were analyzed through the indexation of Bragg peak positions, which fully determined the structures, the space groups and the mean lattice parameter involved within the droplets. The samples were measured at 22 °C through cuvettes of 5 mm thick.

2.4. Spectrofluorimeter measurements

The emission spectra of miniemulsions loaded with Nile Red were measured at indicated time post-preparation using a Jobin Yvon-Horiba Fluorolog 3-22 spectrofluorimeter equipped with a visible detector R928 Hamamatsu. Nile Red was excited at 540 nm and the emission spectra were collected between 560 and 800 nm.

2.5. Cell culture

U-87 MG (Human Glioblastoma) cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 and 5.10^5 cells were seeded every 3–4 days into 25 cm^2 plastic flask. The cells were cultivated in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamax, 1% penicillin/streptomycin, and with 1% of a 100 x non-essential amino acid solution. At these conditions, the U-87 MG cell doubling time was measured at 18 h. Cells were treated at a confluence degree of 60% for cytotoxicity