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Research paper

Lipidic spherulites: Formulation optimisation by paired optical and cryoelectron microscopy

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ABSTRACT

Objective of this study was to assess the various steps leading to spherulite obtention by means of optical and cryoelectron microscopy. The formulation, resting and hydration steps were optimised. Green-based process and organic-based process were compared. It was found that spherulites could be obtained only when two key steps were followed: a prior resting phase of excipients and the shearing stress of the hydrated excipients. Moreover, the new formulation under study formed spherulites in the 100–200 nm range, which is smaller than previously reported spherulites. Such laboratory scale optimised process led the integration of spherulites in a larger number of prospective studies. Indeed, we finally showed that the encapsulated payload of a hydrophobic compound, such as the anti-angiogenic agent fisetin, was increased to a much higher degree than with a liposomal encapsulation.

1. Introduction

Nanoparticles are highly attractive systems able to concentrate drugs and deliver them to a specific tissue. Nanoparticles are valuable drug carriers that are able to include both hydrophilic and hydrophobic drugs, but also proteins and vaccines. Moreover, they can be functionalised for targeting drugs to various tissues and for performing sustained release application [1].

Among nanoparticles, liposomes were the first nano-carriers to be accepted in clinical protocols [2,3]. Through the modification of the drug pharmacokinetic, they allowed a higher payload for a drug to reach the tumour, and, most of all, to reduce the non-specific cytotoxicity of the drug. The first generation of liposomes evolved towards pegylated liposomes, whose clinical use along other drugs has dramatically increased in recent years [4,5].

Chronologically, spherulites were developed at a later stage. The preparation of spherulites was first described by Diat and Roux [6] in 1993. By products from research on crystal lipids, these lipidic assemblies are concentric multilamellar structures, also called onions for their similar aspects. Lamellar phases are composed of a stack of surfactant bilayer separated by aqueous layers. They are obtained through the shearing of that smectic phase. Initially formed as microvesicles, these objects evolved towards change into nanovesicles [7]. The alternate continuous aqueous and lipidic bilayers allow them to incorporate higher amount of hydrophilic or hydrophobic drugs when compared to their multilamellar counterparts, the liposomes. Small drugs [8,9] as well as enzymes [10] or nucleic acids have been encapsulated in spherulites with high efficiency [11,12]. Spherulites for veterinary forms were first marketed by Virbac in 1997 and have been therefore easily scaled-up for industrial applications. They are stable in biological fluids [13], and they can also be targeted [14], which render them excellent candidates for drug sustained release [15]. However, at the laboratory scale, the process is far less reliable. Spherulites are obtained through a formulation in which the amount of the aqueous solvent is finely tuned. When working on small scales, this amount is hardly maintained due to evaporation and losses during the shear step.

In this study, we evaluated the different steps leading to spherulite obtention by means of cross-polarised optical and cryoelectron microscopy. The formulation, resting and hydration steps were optimised. Organic solvent and green solvent-based preparations were compared. Finally, the ability of the spherulites to encapsulate a high hydrophobic drug payload with our optimised formulation and process was evaluated. As spherulites were shown to efficiently encapsulate drugs [7,9], we chose to encapsulate a lipophilic flavonoid derivative, which could possibly be highly encapsulated to a greater extent due to the number of lamellar

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layers in these nano-objects. Fisetin (3,3′,4′,7-tetrahydroxyflavone) has been shown to induce anti-angiogenic effects by stabilisation of endothelial cells in vitro [16] and anticancer activity in vivo [17,18]. Despite the evidence of anticancer activity in vivo, it has been shown that its hydrophobic nature hampers its administration. Recently, we have developed successfully two different formulations for this compound, named nanoemulsions [19] and liposomes [20,21]. Here, we compared the efficacy of fisetin encapsulation in spherulites versus liposomes, as well as the cytotoxicity of encapsulated fisetin.

2. Materials and methods

2.1. Materials

Lipoid® E80 was purchased from Lipoid AG (Ludwigshafen, Germany). According to the specifications, Lipoid® E80 is mainly composed of 80% phosphatidylcholine and 8% phosphatidylethanolamine. Polyoxyethylene sorbitan ester, Polysorbate 80 (Montanox 80) was purchased from Seppic (Paris, France). Water was deionised and purified with a MilliQ system (Millipore, Bedford, MA) then filtered through 0.22 μm (Millipore) prior use. All other reagents were employed as analytical grade. Cyclohexane was purchased from Merck. Fisetin was purchased from Sigma-Aldrich.

2.2. Spherulite preparation

Influence of the process the preparation of spherulites went through a nine-step process. One reference formulation was used for process comparison. The reference formulation included 50/15/35 (m/m/m) of egg lecithin (Lipoïd® 80) polysorbate (Montanox® 80) and distilled water, respectively.

The hydrophilic surfactant and lecithin were precisely weighted and hand mixed to give a homogeneous lipophilic mixture. Then, a first resting time (P1) at 37 °C was applied. At the end of the first resting time, the mixture was hydrated with distilled water, and a second resting time (P2) at 37 °C was applied giving a laminar phase. At the end of the second resting time, the spherulites were obtained by manual shearing of the laminar phase using conic tips and shearing in microtubes (1.5 mL). The spherulites obtained were then dispersed in distilled water for observation. The influence of the two resting times P1 and P2 and manual shearing time were investigated. Resting times of 0–12–24–48 h for P1, 0–24 h for P2 and shearing times of 0–15 minutes, 1 h were investigated.

Freeze-drying was also evaluated using cyclohexane for dissolving lipid components instead of hand mixing. The mixture was then freezed in liquid nitrogen and dried in vacuo under reduced pressure (~0.1 m Bar) during a 12 h-period to eliminate all traces of solvent. This process was applied for fisetin encapsulation.

2.3. Influence of the composition

Different proportions of tensioactive and water were investigated through the optimised process offered by the first investigation using the reference formulation. Egg lecithin was varied from 25% to 60%, polysorbate from 10% to 15% and water from 25% to 60%.

2.4. Spherulite characterisation

2.4.1. Optical microscopy

The concentrated phase was deposed between a slide and a cover slip and observed with a microscope under white light in order to verify the homogeneity of the phase before dispersion. The dispersed spherulites were observed under polarised light with an optical microscope (Olympus BX41).

2.4.2. Cryoelectron transmission microscopy

Spherulite suspensions were 1/100 water diluted and 4 μl deposited on a 200 mesh holey carbon-coated grid (Quantifoil®, EMS, Delta Microscopie, Ayguesvive, France). After blotting with filter paper, the grid was frozen by rapid plunging in liquid ethane and was mounted and inserted in the microscope using a nitrogen cooled side entry Gatan 626 cryo-holder at a temperature of −180 °C. Observations were carried out in a JEOL JEM-2100F transmission electron microscope equipped with an UHR objective lens (Cs: 2 mm, Cc: 2.1 mm, focal length: 1.9 mm), using an accelerating voltage of 200 kV, with the following illumination conditions: alpha 3, spot 3, 100 μm condenser aperture and 70 μm motorised objective aperture. Images were recorded, using the MDS minimum electron dose system (10 electrons per $Å^2$ per sec), with a magnification of 20,000 or 40,000 on Gatan Ultrascan 1000 CCD Camera.

2.4.3. Particle size analysis

The mean particle size and polydispersity index of spherulite suspension were determined by dynamic light scattering (Zetasizer nanoZS®, Malvern, Orsay, France). Refractive index was set at 1.435, and each sample was diluted in water to 1/1000 prior analysis.

2.5. Fisetin encapsulation into spherulites

PS80 (50 mg), E80 (15 mg) and Fisetin (2 mg) were dissolved in cyclohexane (500 μL), lyophilised. H_2O (35 mg) was then added and an immediate manual shearing stress was applied.

2.6. Determination of fisetin encapsulation

The concentration of encapsulated fisetin was evaluated after separation of spherulite and free fisetin on a Sephadex G-25 column by UV spectrophotometry on a Berthold spectrophotometer at 340 nm after dilution of the spherulites in MeOH. The calculated amount of fisetin (mg) per total lipid amount (g) given in Table 3 was based on a calibration curve of fisetin performed in the same solvent.

2.7. Cytotoxicity tests

The murine Lewis lung carcinoma cell line (3LL) was purchased from the American Type Culture Collection (ATCC). The human endothelial cell line (EAhy 926) was obtained from Dr. Edgell [22]. The cell lines were grown in DMEM containing 2 mM L-glutamine, 10% foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (37 °C, 5% CO_2). Cells were plated onto 96-well plates at 5000 cells per well in 100 μL of culture medium. Twenty-four hours after plating, 100 μL of medium containing the compound was added to the wells (in triplicate) containing the cells and incubated for 48 h at 37 °C and 5% CO_2. After a 48 h exposure, cell viability was evaluated using the MTT test, and absorbance was read at 562 nm in a microplate reader (BioKinetics Reader, EL340).

3. Results and discussion

3.1. Method of preparation

Spherulites are obtained from the shearing of a lipidic lamellar phase. The oriented phase is then submitted to dispersion to form spherulites in the 0.2–10 μm diameter range. To form spherulites in a small scale, we detailed each step potentially leading to spherulite obtention and evaluated the impact of each individual step. As represented in scheme 1, spherulites were obtained after the