Development and Validation of Characterization Methods for Lipidots® Multifunctional Platform: a Step forward Industrial Transfer

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ABSTRACT

Lipidots® technology has thrived towards a versatile nanodelivery platform for designing and producing a series of nanoproducts for *in vivo* diagnostic, *in vivo* imaging, activated or non-activated targeted drug delivery. In order to ensure quality of final products, characterization as nanotherapeutics is a key parameter. Thus, we are seeking to implement and validate a panel of characterization methods significantly suitable for Lipidots®. More particularly, we have investigated the lipid quantification, the drug/dye encapsulation, release kinetics and leakage to anticipate the nanocarrier behavior in biological media.

Keywords: lipid nanocarriers, nanomedicine, physical-chemical characterizations, analytical chemistry

1 INTRODUCTION

For eight years, our research group has been developing lipid nanocarriers, known as Lipidots®, as a multifunctional platform for medical applications. A wide range of active pharmaceutical ingredients and contrast imaging agents has been encapsulated into Lipidots® enabling the delivery of therapeutic agents and the monitoring or diagnosis by fluorescence imaging [1-2]. In addition to size and surface charge of the nanomaterials, as key parameters requested by regulatory health agencies, a more deep understanding of nanoparticles composition, morphology, crystallinity, drug loading/localization and drug release kinetics is crucial to meet the requirements of final medicinal nanoproducts. Such extensive characterization in addition to the ability to scale up the manufacturing process of our carriers, allows identifying characterization criteria and specifications to move towards industrial production and approval of the product for the clinical market. Herein, we present a full cascade of physical-chemical characterization methods dedicated specifically to Lipidots®.

2 RESULTS AND DISCUSSION

2.1 Morphology and Physical characterization

Lipidots® are composed of a lipid core, mixture of soybean oil and a wax at different ratios, and a surfactant shell, mixture of phospholipids and PEGylated surfactants. Batches of particles are manufactured by ultrasonication at labscale. Particles with specified diameter can be produced depending on the lipid and surfactants ratios, as previously described [3-5]. For physical characterization of particles, Dynamic Light Scattering (DLS) was used to assess the particle hydrodynamic diameter, as well as their colloidal evolution during storage. We observed very-long term stability under conventional storage (room temperature), even at 40°C. Since the lipid emulsions are known to be destabilized because of coalescence destabilization [6], we evidenced that Ostwald ripening could be prevented by the use of complex mixtures for both the core lipids and the shell surfactants, bringing entropy mixing stabilization to the physico-chemical system [5]. For investigation of internal state of droplets, Differential Scanning Calorimetry (DSC) displayed no evidence of any crystallization event in the particle dispersions, even after prolonged storage at 4°C (up to 8 months), and when the core is made of wax only. This amorphous state should promote long-term encapsulation and homogeneous release of active ingredients from the Lipidots® core. ^{1}H NMR experiments were used to probe the viscosity of the particle core. We demonstrated that core composition and temperature appeared to be key parameters able to control the lipid core viscosity [4]. To characterize the shape of nanodroplets, CryoTEM analysis has been performed showing oblong particles with size distribution in agreement with that measured by DLS. As Flow Field Fractionation (FFF) technique is considered more accurate for polydispersed samples in physiological media, size distribution has been evaluated and corroborated with data obtained from both other techniques.

2.2 Lipidots® Composition

Suppocire NB™ (SC) is a complex mixture of mono- (MGs), di- (DGs) and triglycerides (TGs) of various acyl chain lengths (C8–C18), with an overall hydroxyl value of 20–30%. Super Refined™ Soybean oil (SB) is composed of C16-C18 unsaturated triglycerides. Based on supplier information, Myrj™ S40 (S40) is non-ionic surfactant described as PEGylated stearate with distribution of ethylene oxide (EO) centered around 40 units. As a phospholipid (PL), Lipoid-s75® is mainly composed of

phosphatidylcholine (PC) blended with slight proportion of phosphatidylethanolamine (PE) and lysophosphatidylcholine (LPC) lower than 10 and 3%, respectively.

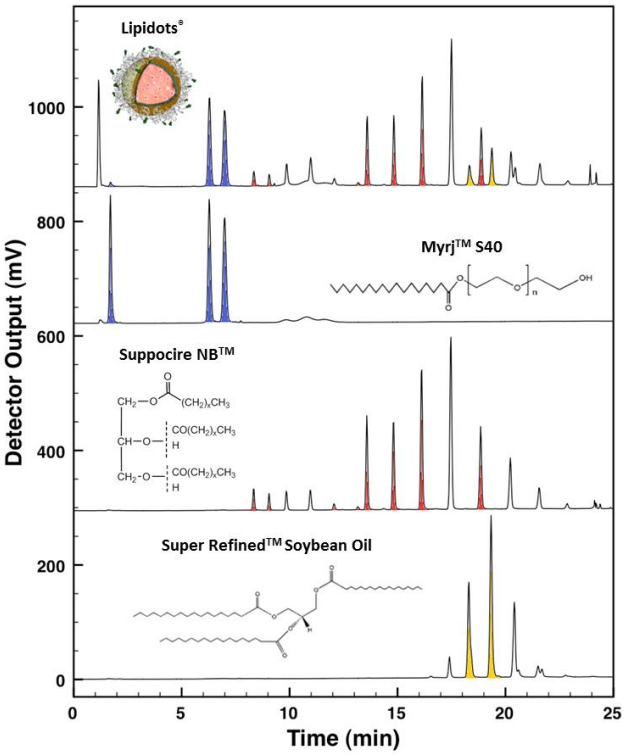

Figure 1: UPLC-ELSD chromatograms of 50 nm-size Lipidots®, Myrj™ S40, Suppocire NB™ and Super Refined™ Soybean oil.

The separation and quantification of lipids belonging to the wax and oil were achieved using reverse-phase (RP) HPLC coupled with Evaporated Light Scaterring Detector (ELSD). Indeed, the RP-HPLC mode is the most important HPLC technique for the analysis of triglycerides mixture. The separation of TGs species is based on both the combined chain lengths of the fatty acyl residues and on the total number of double bonds in the molecule. Normal-phase (NP)-HPLC has been also used for the separation of TGs, however, in most cases only as rather strongly overlapping peak-clusters [7]. Moroever, problems may occur as NP-HPLC separation methods usually use mobile phases consisting of unpolar solvents (like hexane, isooctane or chloroform) as main constituents [8]. Furthermore, it is possible to separate PEG fatty acid esters as single peak per each component with the reversed-phase mode [9]. ELSD was selected as detector since it has low background signal, a non-specific reponse, is compatible with gradient elution and with a broad range of solvents, and has a signal independent of the degree of saturation and chain length of fatty acids or TGs [10]. For PL quantification, Hydrophilic Interaction Liquid Chromatography (HILIC) mode is often recommended when combined several classes of phospholipids [11].

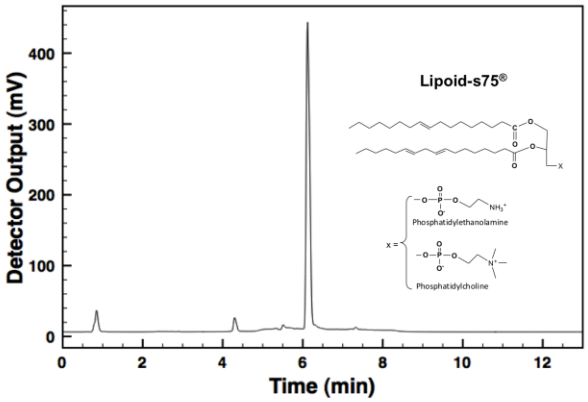

Figure 2: UPLC-ELSD chromatogram of Lipoid-s75® obtained by using HILIC chomatography.

Herein, a RP-HPLC method has been developped for the analysis of Lipidots® mixture. In a single run (Fig. 1), we have sucessfully separated each component of the S40 (blue peaks) and the mixture of MGs, DGs and TGs from SC (red peaks) and SB (yellow peaks). Using mass spectrometry detector, the 3 peaks obtained for S40 surfactant were respectively assigned to PEG-OH, PEG-C16 and PEG-C18. For SB and SC, each peak is actually a cluster of MGs, DGs and TGs, which are identified by mass spectrometry. Once formulated into Lipidots®, most of the lipids can be identified and quantified in a single analysis which run over 30 min with appropriate validated methods. The typical chromatogram obtained for Lipidots® can be used to quantify some individual components incorporated into Lipidots® formulation by using appropriate peaks (colored peaks defined on chromoatogram in Fig. 1). A "fingerprint approach" was established for Lipidots® nanoparticles based on lipid composition and content, enabling to follow up any evolution during manufacturing process and ageing of formulations. Before quantifying PLs from a Lipidots® formulation, S40 components have to be removed using normal phase silica SPE to avoid interferences. Baseline separation of three PLs classes was achieved (Fig. 2), under gradient conditions, within a run time of 13 min, eluting according to decreasing polarity, i.e.: PE > PC > LPC [12]. As previously used for SB, SC and S40 components, a mass spectrometry detection allows identifying the different sub-components of Lipoid-s75®.

2.3 Lipidots® Drug Localization

NMR spectroscopy using proton NOE (Nuclear Overhauser Effect) measurements has been used to localize dyes/drugs in the lipid nanoparticles [2]. This technique can be easily applied to different systems as far as drug/dye signals can be distinguished from Lipidots® signals (generally in range 6-9 ppm). We therefore used this technique to investigate for potential interactions between the CsA with triglycerides (lipid core), phospholipids (particle internal shell) or PEG surfactants (particle external shell). The NOE effect is in first approximation related to

the inter-nuclear distance, since it relies on dipolar interaction between protons. Specific signals representative of the different particle components were chosen (Fig. 3). The specific resonances of the lipid components entering the LNP composition were therefore successively irradiated, and their magnetization, provided that the corresponding proton have dipolar interactions, can be transferred to the –NH peaks of CsA. Whatever the lipid component that was irradiated, NOE transfer occurred to the protons of the amino groups (Fig. 3). It suggests CsA appears located both inside the particle core and in the particle shell. Based on the intensity of the NOE, most of CsA is located inside the particle core.

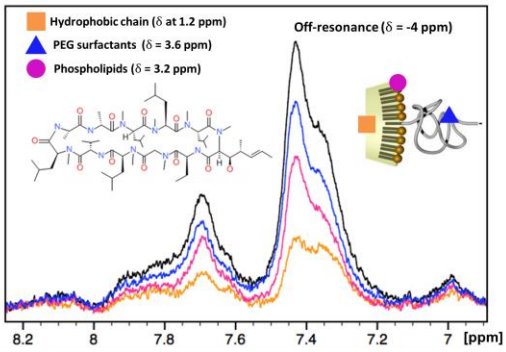

Figure 3: Localization of CsA embedded in 50 nm-size droplets performed by using ^{1}H NMR NOEDIFF spectroscopy.

2.4 Prediction of physiological behavior

Solid phase extraction technique (SPE) has been successfully applied to Lipidots® nanotherapeutic in order to determine leakage kinetics under storage conditions and the drug release profiles in different biological model media. Herein, we give the example of CsA loaded (high-loading) into Lipidots®. Combined with colloidal stability measurements, validated HPLC quantification of free and entrapped drug previously separated by SPE was used to determine the stability of these nanotherapeutic systems at 37°C either in PBS or in DMEM-10%FBS [13, 14].

When stored at 4°C, Hydrodynamic diameter (D_h) and PolyDispersity Index (PDI) remain almost constant for at least 9 weeks. When the nanoparticles are incubated in 1X PBS at 37°C, D_h and PDI do not significantly. In contrast after incubating with DMEM-10%FBS at 37°C the colloidal stability can no longer be guaranteed for 96 h. For at least 24 h, CsA-loaded lipid nanoparticles show great colloidal stability since D_h and PDI remain almost constant for both LNPs. From 48 h, we observed a significant increase of PDI values (>0.3) with a bimodal distribution of particle size.

Thanks to our validated tripartite SPE method, the %free and %entrapped CsA are determined in triplicate at different time-points as illustrated in Fig. 4. When stored at 4°C, the %free and %entrapped CsA remain almost constant over the storage period for both LNPs (not shown). When the nanoparticles are incubated at 37°C in 1X PBS (Fig. 4(A)), the %free and %entrapped CsA of the total concentration are constant over the release period. When the nanoparticles are incubated at 37°C in DMEM-10%FBS, the %free and %entrapped CsA do not also considerably change until 48 h (Fig. 4(B)). For CsA-L50, the %free and %entrapped CsA are equal to 46 and 50% (average until 48 h), respectively. For CsA-L120, %free and %entrapped CsA are respectively equal to 52 and 44% (average until 48 h). Further investigations have also demonstrated that the %free and %entrapped CsA were dilution-dependent. Indeed, we found that %free and %entrapped CsA were respectively 32 and 65%, for CsA-L50 at a concentration of 10 mg/mL.

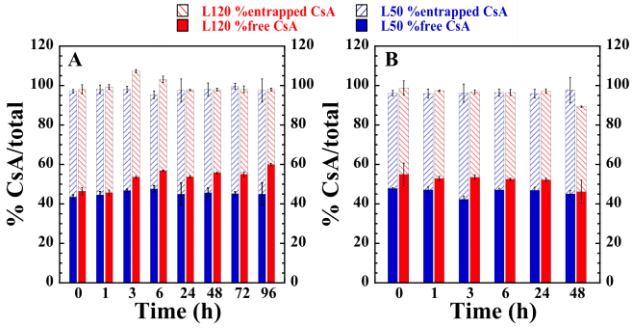

Figure 4: Drug release profiles of 50 nm and 120 nm-size CsA loaded nanodroplets performed at 37°C in 1X PBS (A) or in DMEM-10%FBS (B).

The release profile is similar whatever the buffer/medium used, thus indicating a biphasic mode of release. This suggests that the CsA may be loaded not only in the lipid core, but also in the shell responsible of the observed leakage.

3 CONCLUSION

Extensive characterization dedicated specifically to Lipidots® as a nanomedicine allows identifying characterization criteria and specifications to move towards industrial production and approval of the product for the clinical market. Very recently our group has begun to coordinate the European nanomedicine characterization platform (EU-NCL). Connected to a trans-disciplinary testing infrastructure, this platform will propose a cascade of assays for the pre-clinical characterization of medical nanoparticles, promoting the translation of nanoproducts towards clinical evaluation more rapidly and for lower cost and also paving the way for future pre-normative activities in order to regulate the development of nanomedicines.

4 EXPERIMENTAL SECTION

4.1 Lipidots® Processing

The nanoemulsion processing has already been detailed elsewhere [3-5, 15]. Briefly, lipid phase is prepared by

mixing solid (Suppocire NC, Gattefossé) and/or liquid (Super-refined Soybean oil, Croda) glycerides, Lipoid s75 (Lipoid GmbH), and eventually the compound to be loaded in the Lipidots®. The aqueous phase is composed of Myrj™ S40 (Croda) and aqueous buffer (usually 1X PBS). After homogeneization at 45°C, both phases are crudely mixed and sonication cycles are performed for 5 minutes. Non-encapsulated components are separated by overnight dialysis (MWCO: 12 kDa). Lipidots® dispersions are filtered through a 0.22 µm cellulose Millipore membrane before use. CsA free drug was incorporated into lipid core by solvent-mediated dissolution from 75 mg/mL CsA solution, as previously described [13]. CsA drug was loaded until a concentration of 3825 µg/mL and 5079 µg/mL (for 60 mg/mL of lipids), respectively for 50-nm and 120-nm nanodroplets. Due to low solubility of CsA as free drug in aqueous buffer, dialysis purification was extended until 72 h with extensive changes of buffers.

4.2 Morphology and structure measurements

The hydrodynamic diameter are measured with a Malvern Zeta Sizer Nano instrument (NanoZS, Malvern, UK) in 0.1 X PBS buffer. DSC thermograms and ^{1}H NMR spectra have respectively been recorded on a TA Q200 system (TA instrument, France) and a Bruker Avance DPX 500 spectrometer (Bruker, Germany), operating at 500 MHz for proton.

4.3 Determination of Lipidots® composition

The chromatographic analysis of the Lipidots® components was performed using an Acquity UPLC® H-Class system (Waters) coupled with an Alltech 3300 Evaporating Light Scattering detector (ELSD, Grace). Separation of the different compounds was achieved either with CORTECS RP-18 or HILIC (1.6 µm, 150 x 2.1 mm) columns. In reversed-phase mode, the separation was performed with a gradient program of water, methanol and a mixture of isopropanol/acetonitrile : 75/25 (v/v). Samples was diluted with a mixture of $CHCl_3$/MeOH:2/1 (v/v). The drift tube was maintained at 45°C with a flow of N_2 fixed at 2.0 L/min. In HILIC mode, the separation was performed with a gradient program of acetonitrile/ammonium formate, 10 mM, pH3: 95/5 and 50/50 (v/v). Sample (Lipoid-s75®) was dissolved in methanol. The drift tube was maintained at 35°C with a flow of N_2 fixed at 1.3 L/min. Whatever the chomatography mode, the injected volume was 5 µL and the column temperature was fixed at 40°C.

4.4 NMR spectroscopy for CsA localization

^{1}H NMR Nuclear Overhauser Enhancements difference (NOEDIFF) spectra of CsA-loaded lipid nanoparticles were recorded using the spectrometer described above. To avoid signal saturation arising from the intense proton signals of the bulk nanodroplets component, the basic sequence was modified by changing the last reading in proton impulsion in the sequence by a selective pulse. This selective pulse was calibrated to excite selectively a spectral range from 6 to 9 ppm, which corresponds to NMR signals of the proton in –NH group. Then a series of spectra were recorded by irradiating the specific peaks of interest, corresponding to each lipid nanoparticles component. A control spectrum (off-resonance) was also recorded by performing the same experiment with an irradiation at a frequency outside the range of any NMR signals (-2000 Hz). Prior to the measurement, 300 µL of deuterium oxide was mixed with 600 µL of nanoparticle suspension at a concentration of 60 mg/mL of lipids.

4.5 Leakage and release studies of CsA

The protocol used for the determination of CsA release and leakage was based on our previous tripartite SPE method [13, 14]. The separation between the entrapped CsA and free CsA was performed by using SPE-cartridges Oasis® HLB (6 mL, 150 mg, 80 Å, 60 µm). The leakage study was achieved for samples of CsA-loaded 50 nm and 120 nm-size Lipidots®, which were stored in 1X PBS at 4°C at a nanoparticle concentration of 60 mg/mL of lipids. Starting from the freshly prepared suspensions, leakage study was recorded weekly during 9 weeks. Release study was achieved at 37°C under gentle stirring at a nanoparticle concentration of 2 mg/mL either in 1X PBS or in DMEM containing 10% of FBS. Leakage and release profiles were determined by implementing SPE procedure following by HPLC-UV quantification. Validation of the tripartite SPE method has been performed according to ICH guidelines Q2(R1) [16] for both LNPs either in PBS or in DMEM-10%FBS. Size distribution of Lipidots® was monitored by DLS during the release and leakage study.

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