European Journal of Pharmaceutics and Biopharmaceutics 110 (2017) 31–38

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Determination of nanostructures and drug distribution in lipid nanoparticles by single molecule microscopy

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ARTICLE INFO

Article history:
Received 12 May 2016
Revised 27 October 2016
Accepted in revised form 28 October 2016
Available online 31 October 2016

Keywords:
Lipid nanoparticles
Nanostructured lipid carriers
Single particle tracking
TIRF microscopy
Fluorescence superresolution microscopy
Drug delivery systems
Drug loading

ABSTRACT

Drug loading capacity in nanostructured lipid carriers (NLC) depends on the formation of nanostructures within the lipid matrix. However, investigation of these nanostructures with sizes below the diffraction limit of visible light is quite challenging. Thus, until now the determination of structures and drug distribution within NLCs was not possible. Therefore, we aimed at developing a method to visualize the nanostructures within the lipid carriers. Model NLCs loaded with a lipophilic fluorescent drug mimetic, ATTO-Oxa12, were produced and investigated by single-molecule tracking and localization-based superresolution microscopy. Results revealed spherical ATTO-Oxa12-filled nanostructures with diameters of ~70 nm and 120–130 nm, both smaller than the NLC size (~160 nm). The ATTO-Oxa12 diffusion constant was calculated from the single-molecule traces ($D \geqslant 1\ \mu m^2/s$) and indicated the distribution of the model drug in the oily component. Together these data suggest the existence of drug-loaded oily nanocompartments, which could fill up to ~50% of the model NLCs' volume. In conclusion, a novel tool based on single-molecule microscopy is now available that allows for the precise determination of drug distribution and the characterization of lipid nanostructures, information that is paramount for optimizing lipid nanoparticle formulations.

1. Introduction

Nanostructured lipid carriers (NLCs) and their predecessors, the solid lipid nanoparticles (SLN), are nanoparticles made from lipids and were developed to increase the bioavailability of lipophilic actives [1]. Whereas the lipid matrix of SLN consists of only one solid lipid, the matrix of the NLC is made from a blend of a solid and a liquid lipid (solid and liquid referring to the respective state of the lipid at room or body temperature). This leads to the formation of possible 'nanostructures' within the lipid matrix [1]. These nanostructures increase the drug loading capacity and influence the distribution and release profile of the drug [1]. Hence, these structures play a key role in defining the in vivo performance of NLCs. For optimal formulation development, i.e. tailoring the release profile to a specific application, it is therefore of fundamental importance to gain insight into these nanostructures. This should help to better understand the drug distribution within the particle as well as to get more detailed information on drug release.

Obtaining this kind of information, however, depends on the measurement techniques available. Dynamic light scattering (DLS) and laser diffraction (LD) are routine measurement methods to determine the size of NLCs although they have low resolving power and the determined sizes are affected by either large aggregates or inhomogeneously formed particles [2]. Unfortunately, no information on the internal structure of NLCs can be obtained from DLS and LD. Higher structural resolution is available from electron microscopy (EM) methods. Thus, the NLC particle morphology and internal structure has been investigated by different EM techniques [3–5]. Here, depending on the formulation, the EM method, and the measurement conditions, different results were obtained. CryoEM measurements identified NLC platelets with nanostructures attached to the NLC surface [4,6,7], while with transmission electron microscopy (TEM) at room temperature spherical NLC particles were seen, but nanostructures were not detected [8–10]. These varying results could be due to differences in sample treatment prior to CryoEM and TEM, or to variations in formulation and production procedures of the analyzed NLCs. Formulation and production are both thought to influence the arrangement of solid and liquid lipids within the NLCs [1,11]. Thus, due to the scarcely available experimental evidence several different hypotheses

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http://dx.doi.org/10.1016/j.ejpb.2016.10.020
0939-6411/

about nanostructures within NLCs and the resulting drug distribution were put forward in the past [12,13]. It is proposed that, depending on the NLC formulation and production conditions, the liquid lipid (oil) can be homogeneously distributed within the particle or localized in nanocompartments. There could either be multiple nanocompartments, a single core or shell, or the nanocompartment could only be associated to or even separated from the particle itself (Fig. 1).

To solve this question, we present a novel experimental approach based on single-molecule fluorescence microscopy to investigate the morphology of lipid nanocarriers, directly visualizing the drug distribution within the NLCs on the nanometer scale. Fluorescence-based techniques have been shown to be extremely powerful tools in the investigation of nanoparticles. For the characterization in terms of molecular interactions, dynamics, structures and transport properties advanced fluorescence techniques like time-resolved fluorescence depolarization, fluorescence correlation spectroscopy, raster image correlation spectroscopy and fluorescence lifetime imaging microscopy have been used recently [14–21]. Here, we adapted existing methods such as single particle tracking (SPT) [22–24] and stochastic optical reconstruction microscopy (superresolution microscopy) [25–27] to the investigation of NLCs. SPT not only reveals the diffusive properties of single molecules but also can be used to visualize hot-spots of interactions in a so-called visit map analysis [28,29]. Stochastic localization based superresolution techniques may visualize structures below the diffraction limit in optical microscopy [22,25,27,30]. Using these methods we characterized NLCs that were produced by hot high pressure homogenization.

2. Materials and methods

2.1. Production of NLCs loaded with a fluorescent drug mimetic

NLCs consisting of Miglyol® 812 (Caelo, Germany) and Dynasan® 118 (Sasol, Germany) were loaded with the lipophilic fluorescent dye ATTO-Oxa12 NHS ester (ATTO-TEC, Germany) as drug mimetic. NLCs consisting of Dynasan® 118 (solid lipid), and Miglyol® 812 (medium chain triglycerides) as matrix lipids were produced by hot high pressure homogenization. The dispersion was stabilized by 2% (w/w) Plantacare® 818 (C8–16 fatty alcohol glycoside, Cognis, Germany). For dye loading 0.02 mg of ATTO-Oxa12 (Fig. 2) was dispersed within 0.4 mg Miglyol® 812, the liquid oil. The solution obtained was admixed to the molten solid lipid (Dynasan®). Finally, the aqueous phase, containing the stabilizer and water, was heated to the temperature of the liquid phase and was then poured into the lipid phase. The mixture was stirred for 1 min using an Ultra Turrax T25 (Janke & Kunkel, Germany). The obtained pre-emulsion was subjected to hot high pressure homogenization, using an LAB 40 (APV, Germany) in discontinuous mode, by applying 3 homogenization cycles at 500 bar at a temperature of 80 °C. The compositions are given in Table 1. For further analysis the samples were diluted to yield samples containing either 0.1% or 0.001% lipid.

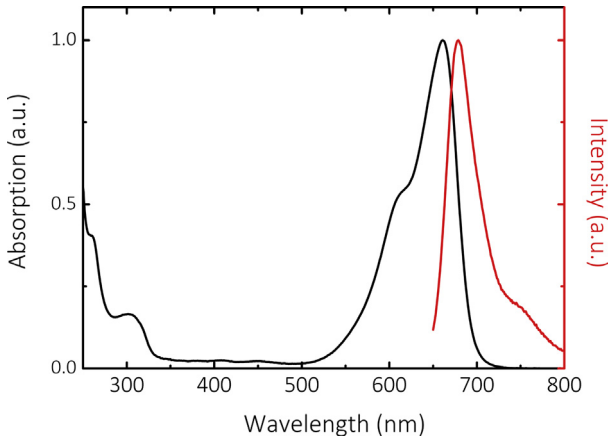

Fig. 2. Absorption (black) and fluorescence emission (red) spectra of Atto-Oxa12 in H_2O. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Dynamic light scattering and laser diffractometry

The NLC size was analyzed by dynamic light scattering (DLS) and laser diffractometry (LD). DLS measurements were performed by using a Zetasizer ZS nano (Malvern, UK) and results were analyzed using the general purpose mode. LD measurements were performed using a Mastersizer 2000 (Malvern, UK). Results were analyzed by using the Mie-theory with the optical parameters 1.456 (real refractive index) and 0.001 (imaginary refractive index).

2.3. Sample preparation for single molecule experiments

A requirement for the determination of size and internal structures of the NLCs using single molecule microscopy is that the dye movement is not superimposed by motion of NLCs. To this end the NLCs needed to be immobilized on the cover glass. A common method in the literature for immobilizing NLCs, e.g. to determine their size by electron microscopy, is to place a drop of solution onto the sample holder and to let the solute evaporate [8–10,31]. Here, a 10 µl drop of aqueous NLC solution (0.1% NLC, 00001% ATTO-Oxa12) was pipetted onto a meticulously cleaned cover slip and the water was left to evaporate. To prevent any contamination of the sample, the cover slip was kept in a cleaned glass container with the lid only slightly open and placed inside a sealable plastic bag. Samples were measured at the earliest three days after

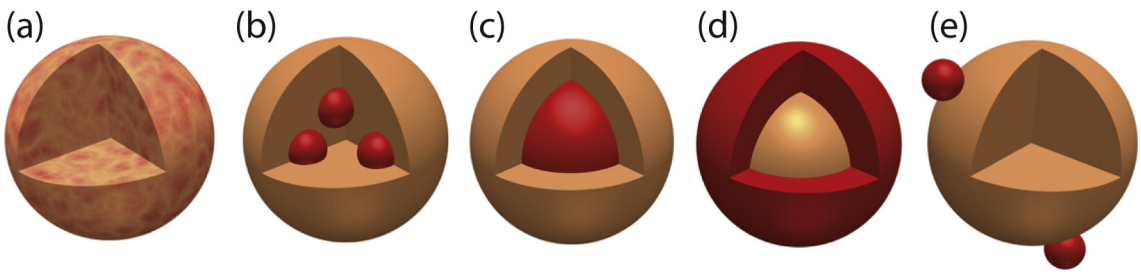

Fig. 1. Possible arrangements of the liquid and solid lipid components within the lipid matrix of NLCs. (a) Homogeneous distribution of the liquid lipid within the lipid matrix. (b) Liquid lipid containing subdomains within the solid phase. (c) Single core of liquid lipid encapsulated in the solid phase. (d) Single core of solid lipid encapsulated by the oily lipid. (e) Liquid lipid nanocompartments associated with the surface of the solid phase.