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Size fractionation and size characterization of nanoemulsions of lipid droplets and large unilamellar lipid vesicles by asymmetric-flow field-flow fractionation/multi-angle light scattering and dynamic light scattering

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ABSTRACT

Asymmetric-flow field-flow fractionation technique coupled to a multi-angle light-scattering detector (AF4-MALS) was used together with dynamic light-scattering (DLS) in batch mode and transmission electron microscopy (TEM) to study the size characteristics of the trioleoylglycerol lipid droplets covered by a monolayer of sphingomyelin and cholesterol, in water phase. These lipid droplet nanoemulsions (LD) were formed by ultrasonication. In parallel, the size characteristics of large unilamellar lipid vesicles (LUV) prepared by extrusion and composed of sphingomyelin and cholesterol were determined. LD and LUV were prepared at two different molar ratios (1/1, 4/1) of sphingomyelin and cholesterol. In AF4-MALS, various cross-flow conditions and mobile phase compositions were tested to optimize the separation of LD or LUV particles. The particle radii, R, as well as the root-mean-square radii, R_{rms}, of LD and LUV were determined by AF4-MALS, whereas the hydrodynamic radii, R_h, were obtained by DLS. TEM visualization revealed round shape particles of LD and LUV.

1. Introduction

Naturally occurring lipid droplets and artificially formed oil-in-water droplets, forming emulsions, share several physico-chemical characteristics in common. Essentially, both classes of colloidal in-water dispersions are thermodynamically metastable and are kinetically stabilized by a surfactant [1–4]. Specifically, lipid droplets are ubiquitous intracellular organelles [1,2], composed of a core of neutral lipids, mostly triacylglycerols and cholesteryl esters, enveloped by an amphiphilic lipid monolayer that includes specific proteins. Amphiphilic lipids including sterols, located preferentially on the surface of lipid droplets, decrease surface tension and thus increase their stability in water phase [5,6]. These cellular organelles are involved in lipid metabolism and are considered to be the storage sites of neutral lipids, and to play a role in whole-body energy homeostasis [7]. In artificial lipid droplet nanoemulsions (LD), lipid droplets are formed by high-energy emulsification methods such as ultrasonication or high pressure homogenization [3,8,9].

In pharmacy, nanoemulsions of lipid droplets (LD) and lipid vesicles are used in drug delivery systems, most recognizable in dermatological applications and gene therapy [10,11]. Their size and size distribution are key factors affecting their pharmacodynamics [12]. Similarly, biophysical and biological properties of intracellular lipid droplets are critically modulated by their size [6].

Biophysical characteristics of large lipid vesicles (LUV) with one (unilamellar) or several (multilamellar) lipid bilayers have been extensively studied. The size and size distribution of LUV have been characterized by dynamic light-scattering (DLS), static light-scattering (SLS) [13–17], microscopic techniques (fluorescence microscopy, transmission electron microscopy, scanning electron microscopy, freeze-fracture electron microscopy [18–21]), analytical ultracentrifugation [18], size-exclusion chromatography [22], and flow field-fractionation (FFF) techniques [23–30], whereas

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the size and size distribution of lipid and oil-in-water emulsion droplets have been generally characterized by electron microscopy [2,9,31], DLS [32–34], and SLS [35,36].

Size characterization of LUV and LD by light-scattering techniques is preferred since the measurements are performed in suspension. DLS is continuing to be the method of choice since the pivotal study of van Zanten and Monbouquette [16] on phosphatidylcholine/sodium cholate vesicles. However, asymmetric-flow field-flow fractionation technique (AF4) coupled to a multi-angle light-scattering (MALS) enables particles' separation by size and determination of size (radius), size distribution as well as the total number and number density distribution of particles [24–28]. The interest in field-flow based fractionation techniques for the separation and analysis of macromolecules and supramolecular assemblies is steadily increasing [37].

This study focuses on the size characterization of LD, composed of a trioleoylglycerol (TOG) core coated with a sphingomyelin (SM)/cholesterol (Chol) monolayer, using AF4-MALS, batch-DLS, and TEM. In parallel, we studied LUV made of SM and Chol as a biophysically well-characterized vesicular species. A combination of SM and Chol was chosen since these lipids are essential for formation of specific lipid domains such as lipid rafts in artificial lipid membranes or biological membranes, where they have multiple biologically important functions [38]. Moreover, LD covered by combined sphingomyelin and cholesterol have been poorly characterized in contrast to respective SM/Chol vesicles. Our study was furthermore stimulated by the idea that nanoemulsions of lipid droplets bearing a polar lipid monolayer surface may serve as a useful lipid membrane model complementary to a vesicle lipid bilayer or lipid monolayer formed at the water/air interface. Indeed, our preliminary studies of lipid-binding proteins suggested that certain proteins may discriminate between a SM/Chol surface located either on vesicles or lipid droplets.

2. Experimental

2.1. Large unilamellar lipid vesicle (LUV) preparation

LUV were prepared from porcine brain sphingomyelin (SM) and wool grass cholesterol (Chol) (Avanti Polar Lipids, Alabaster, USA) as described before [39,40]. Briefly, the lipids were dissolved in chloroform/methanol mixture (3/1, v/v) at a SM/Chol molar ratio of 1/1 or 4/1. The solvent was removed by rotary evaporation under reduced pressure (40 mbar) for 4 h. Multilamellar vesicles were formed by hydrating the lipid film in the following solvents: (i) 10 mM Hepes, pH 8.0; (ii) 10 mM Hepes with 50 mM NaCl, pH 8.0; and (iii) 50 mM sodium nitrate, to obtain finally concentration of 10 mM lipids. The hydrated lipids were vortexed with added glass beads until the lipids were removed from the flask surface. The dispersed lipids were subjected to eight cycles of freezing in liquid nitrogen and thawing. Finally, LUV were made by extrusion of multilamellar vesicles through a polycarbonate 100 nm membrane mounted in a mini-extruder (Avestin, Ottava, Canada). Passages (31) were applied at the temperature exceeding 41 °C (the phase transition temperature of SM) to obtain LUV. Prior to AF4-MALS measurements the LUV dispersions were diluted to approx. 0.5 mg/mL lipid concentration.

2.2. Lipid droplet nanoemulsions (LD) preparation

LD were prepared from trioleoylglycerol (TOG) (≥99%, Sigma–Aldrich, Munich, Germany), porcine brain SM, and wool grass Chol (Avanti Polar Lipids, Alabaster, USA) as described in detail before [41]. Briefly, the lipids at a SM/Chol/TOG molar ratio of 1/1/4.7 or 4/1/11.7 were dissolved in chloroform/methanol (3/1, v/v), and the lipid dispersions were prepared as described above for multilamellar lipid vesicles. Thereafter, the protocol was followed by 30 min sonication using a Vibracell Ultrasonic Disintegrator (Sonics&Materials, Newtown, USA) at 35% amplitude on ice, and double centrifugation at 30,000 and 20,000 × *g* for 1 h at room temperature. The upper layer with floating LD was carefully separated from the rest of the suspension containing denser lipid droplets [41], and collected in an Eppendorf tube. Prior to AF4-MALS measurements the LD dispersions were diluted to approx. 0.5 mg/mL lipid concentration.

2.3. Transmission electron microscopy (TEM)

A 3.0 μL of lipid sample in 10 mM Hepes, pH 8.0, was left for 60 s on a formvar and carbon coated copper grid, and excess solution was removed with Whatman filter paper. To reduce salt effect, three droplets of deionized water were adhered to the grid for 1–2 s. After removal of the excess fluid, the grid was negatively stained with 1% (w/v) uranyl acetate, with the excess staining solution blotted and the grid allowed to dry. The specimens were examined with a Philips CM 100 electron microscope (FEI, Eindhoven, the Netherlands), operating at 80 kV. The data were collected with an ORIUS SC 200 CCD camera (Gatan Inc., Pleasanton, USA) using Digital Micrograph Software (Gatan Inc., Pleasanton, USA). The particle radius was measured solely on lipid particles well separated from each other (non-crowded particles) since they preserved round-shaped structure. Average radius (R_{TEM}) and standard deviation (s.d.) were calculated with a Statistica 8.0 software package (StatSoft, Hamburg, Germany).

2.4. Asymmetric-flow field-flow fractionation coupled to a multi-angle light-scattering detector (AF4-MALS)

AF4 separations were performed by Eclipse3+ system (Wyatt Technology Europe GmbH, Dernbach, Germany) using a trapezoidal long channel LC 240 mm, a 350 μm spacer, and a Nadir Cellulose RC (Regenerated Cellulose) PCK5 membrane with a 10 kDa cut-off (Wyatt Technology Europe, Dernbach, Europe). The system also included an isocratic pump, an on-line vacuum degasser and an autosampler (all Agilent 1260, Agilent Technologies, Santa Clara, USA). The fractionated particles were detected with an on-line UV detector at 280 nm (Agilent Technologies), and a multi-angle light-scattering (MALS) detector (DAWN-HELEOS, Wyatt Technology Corporation, Santa Barbara, USA) with a GaAs laser (λ_o = 658 nm), calibrated with toluene and normalized with bovine serum albumin protein as an isotropic scatterer standard. The separations were carried out at room temperature in three different mobile phases (Table 1). The mobile phases were supplemented with sodium azide (NaN_3) (0.02%, w/v; Sigma–Aldrich, Munich, Germany) as a bactericide. The mobile phases were filtered through a Nylon 6,6 membrane with a pore-size of 0.45 μm (Sigma–Aldrich, Munich, Germany). An additional filter (0.1-μm pore size, Durapore membrane) was included in the system (PEEK Inline Filter Holder) after the HPLC pump.

A focusing/injection step of 4 min with a focus-flow rate of 1.5 mL/min and an injection-flow rate of 0.2 mL/min was

Table 1
List of mobile phase compositions used for AF4-MALS experiments.

	Mobile phase composition
MP-I	10 mM Hepes, pH 8.0
MP-II	10 mM Hepes with 50 mM NaCl, pH 8.0
MP-III	50 mM $NaNO_3$

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