International Journal of Pharmaceutics 523 (2017) 320–326

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Analytical ultracentrifugation for analysis of doxorubicin loaded liposomes

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ARTICLE INFO

Article history:
Received 2 February 2017
Received in revised form 20 March 2017
Accepted 21 March 2017
Available online 22 March 2017

Chemical compounds studied in this article:
Doxorubicin hydrochloride (PubChem CID: 443939)

Keywords:
Analytical ultracentrifugation
AUC
Liposome
Nanomedicine
Free drug
Size distribution

ABSTRACT

Analytical ultracentrifugation (AUC) is a powerful tool for the study of particle size distributions and interactions with high accuracy and resolution. In this work, we show how the analysis of sedimentation velocity data from the AUC can be used to characterize nanocarrier drug delivery systems used in nanomedicine. Nanocarrier size distribution and the ratio of free versus nanoparticle-encapsulated drug in a commercially available liposomal doxorubicin formulation are determined using interference and absorbance based AUC measurements and compared with results generated with conventional techniques. Additionally, the potential of AUC in measuring particle density and the detection of nanocarrier sub-populations is discussed as well. The unique capability of AUC in providing reliable data for size and composition in a single measurement and without complex sample preparation makes this characterization technique a promising tool both in nanomedicine product development and quality control.

1. Introduction

Size makes the most obvious difference between nanomedicine products and conventional medicine. Indeed, carrier size distribution is identified as one of the key parameters determining the biodistribution and pharmacokinetics (PK) of nanoformulated drugs. Being crystallized, encapsulated or linked to a particle in the nano size-range often ensures a molecule better barrier penetration, improved bioavailability or more efficient accumulation in the target tissue as compared to the conventional free drug (Angi et al., 2014; Desai, 2012; Fuhrmann et al., 2014; Ross et al., 2015; Szabo and Zelko, 2015). Other features, like coatings inhibiting opsonization were also proven to be creditable for enhanced efficacy of nano-pharmaceuticals (Vonarbourg et al., 2006). In cancer treatment, direct targeting of the tumor tissue by linking antibodies or receptor ligands to the surface of the nanoparticles is identified – and sometimes disputed – as a trend in nanomedicine research (Lammers et al., 2016; van der Meel et al., 2013). However, the complications of loading active compounds in some promising nano-carriers (Raemdonck and De Smedt, 2015) or the most prominent success stories of nanomedicine, like Abraxane (Sparreboom et al., 2005) or Doxil (Barenholz, 2012) still highlight the significance of the free versus encapsulated (or bound) drug ratio in the biodistribution and pharmacokinetics of nanoformulated products. For example, in the case of Doxil, the decreased cumulative concentration-dependent cardiotoxicity of doxorubicin in pegylated liposomal formulation is attributed to the longer half-life of the drug inside the liposomes and decreased myocardial concentration of the free drug (Rahman et al., 2007; Tahover et al., 2015). In this context, the low concentration of the free cytotoxic drug in the nanomedicine product becomes a warranty for drug safety.

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http://dx.doi.org/10.1016/j.ijpharm.2017.03.046

All the above mentioned parameters: size distribution, composition, coating, functionalization, drug loading, free versus encapsulated drug content are extremely important characteristics not only during the design and pilot experimental phase but also during the later production phases of nanomaterials. Measurement of these parameters is critical in scale-up processes, in monitoring batch to batch variations as part of an internal quality system as well as during drug safety evaluations by an external authority (Diou et al., 2015; Landesman-Milo and Peer, 2016; Ragelle et al., 2016).

Among the size measurement methods, dynamic light scattering (DLS) is the most widespread technique applied in nanomedicine research. The main drawback of batch mode DLS measurement, i.e. light scattering by larger particles tends to hide the smaller particle populations, can be overcome by applying it after a size fractionation separation step such as asymmetric field flow fractionation (AF4) (Iavicoli et al., 2015). However, the measurement following AF4 particle separation usually needs much longer analysis times, includes particle specific method development and frequently also dilutes the concentration of particles below the limit of detection especially for smaller particles.

While simple UV-spectrophotometric procedures also exist for determination of DoxHCl concentration (Manasa et al., 2013), liquid chromatographic (LC) methods are the golden standards in the analysis of drug concentration in pharmaceutical products and biological media (Maudens et al., 2009). Various detectors (from UV–vis absorbance to mass spectrometry) coupled to the LC system provide a wide range of specific quantification techniques for the different active compounds – after LC separation method development and calibration.

Although Analytical ultracentrifugation (AUC) is a method mostly applied in protein size measurements and kinetic studies (Brown and Schuck, 2006; Dam et al., 2005; Schuck, 2000) it has also great potential in measuring particle size up to the micrometer range – depending on the density of nanoparticles (Carney et al., 2011; Wohlleben, 2012). AUC is a first-principles based technique, requiring no calibration by means of a particle size standard and determining particle size from the sedimentation speed of the components of a suspension (Mittal et al., 2010; Planken and Colfen, 2010). Modern AUC instruments are capable to monitor sedimentation of polymeric nanoparticles and liposomes in a water based suspension using both absorbance and/or refractive index (RI) detector(s). In case of known densities, the measured sedimentation coefficient distributions can be converted to mass based size distributions. However, even direct comparison of sedimentation coefficient distributions can provide information about batch to batch variability or drug loading.

As the molecular mass of a typical small the drug molecule is generally about 5 orders of magnitude lower than the mass of an encapsulating liposome, sedimentation speed of the free drug is negligible compared to the sedimentation speed of the nanoparticles. In case of molecules absorbing in the UV–vis spectral region, this results in a practically stable time-independent and radius-independent free-drug background absorbance at properly chosen AUC rotational speed. The ratio of this “background” signal to the signal corresponding to the sedimenting fraction(s) provides information about the ratio of free absorbing material outside the liposomes.

In this work, we describe the applicability and limitations of AUC as simple analytical method in the characterization of nanomedicine products. We measure free vs. encapsulated drug ratio and particle size distribution by AUC and demonstrate that the generated results match well with reference method results (HPLC analysis and DLS).

2. Materials and methods

2.1. Materials

Doxorubicin containing Dox-NP™ and control empty liposomes were purchased from Avanti PolarLipids. According to the manufacturer's specification, nominal doxorubicin concentration was about 2 mg/mL with 97.3% encapsulation in the loaded liposomes. Doxorubicin hydrochloride (DoxHCl, European Pharmacopeia reference standard) and all other chemicals were purchased form Sigma Aldrich. Phosphate buffered saline solution (PBS) was filtered through a 0.2 μm syringe filter (Millipore) before use.

2.2. UV–vis spectroscopy

UV–vis (UVVis) spectra of the Dox-NP™ suspension and the free drug were recorded in PBS using 0.5 mL quartz cuvettes and a Nicolet Evolution 300 (Thermo) UV–vis spectrophotometer.

2.3. Particle size measurements

Batch mode DLS measurements were performed using a Malvern Zetasizer Nano-ZS instrument equipped with a 633 nm HeNe laser. The original liposome suspensions were diluted 100 times in PBS and equilibrated for 5 min before the measurements at 25 °C. Size distribution results were generated by averaging 10 consecutive measurements of 12 times 10 s runs.

Online coupled FFF-UVVis-MALS-DLS measurements were performed using a Postnova AF4 asymmetric field flow fractionation system connected to a Malvern Zetasizer Nano-ZS instrument and a Wyatt Dawn Heleos multi angle light scattering (MALS) detector. The diluted liposome suspensions were injected through a 20 μL loop into the FFF channel (350 μm spacer, 10 kDa regenerated cellulose membrane). PBS (pH 7.4) was applied as mobile phase at 0.5 mL/min flow, 1.3 mL/min crossflow and exponentially decreasing crossflow profile and total running times of 60–80 min. Absorbance of the eluted fractions was monitored at 490 nm and 280 nm for the loaded and empty liposomes, respectively. Hydrodynamic and geometric size of the particles was determined at the maximum of the elugram from the on-line DLS and MALS measurements, respectively.

Cryo-TEM images were taken using a Tecnai Osiris (FEI) transmission electron microscope at 200 kV. Before sample preparation Dox-NP™ suspension was 1:1 diluted in MilliQ water, while the suspension of control liposomes was loaded on the grid and analyzed without any dilution. A sample aliquot of 2 μL was spot on Agar C-166-3 lacey carbon grid. The sample was automatically vitrified by using a Vitrobot (FEI). The raw images were analyzed manually counting 300 particles for each sample, and collecting size (Feret diameter) and shape information by Image J. Size distributions were plotted by using Origin v 8.0. Average size and standard deviation were calculated by fitting the two histograms with a normal distribution curves.

2.4. HPLC analysis

Analysis of doxorubicin concentration was conducted using a Waters liquid chromatographic system composed of a 1525 binary pump and a 2487 dual lambda absorption detector set at 234 nm wavelength. Chromatographic separation was achieved using a Phenomenex Kinetex 2.6 μ XB-C18 100A (75 × 4.6 mm) column. The mobile phase was composed of (A) 0.1% trifluoroacetic acid (TFA) in ultrapure water and (B) 0.1% TFA in acetonitrile. The gradient method was (v/v): 15% B (3 min), 15–100% B (10 min), 100% B (3 min), 100-15% B (4 min), 15% B (10 min), with total