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Research Paper

Heating treatments affect the thermal behaviour of doxorubicin loaded in PEGylated liposomes

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ABSTRACT

Doxil® is a stealth marketed PEGylated liposomal formulation, containing the anticancer drug doxorubicin. After loading via a pH gradient, fibrillar supramolecular structures of doxorubicin sulfate originates inside the core of the liposomes. Recently, the crystallinity of doxorubicin sulfate has been confirmed by high-resolution calorimetry. However, no detailed information are available on the nature of doxorubicin sulfate nanocrystals and on the effect of different thermal treatments. Thus, the aim of this work was to characterize the thermal behaviour of Doxil® in comparison to the unloaded liposomes using microcalorimetry, dynamic light scattering and high-resolution ultrasound spectroscopy (HR-US). Different thermal programmes were applied with the aim to highlight the effect of the treatments on the formulation. The used techniques confirmed the ordered state of doxorubicin nanocrystals inside PEGylated liposomes. Particularly, microcalorimetry and HR-US highlighted the changes in the thermal behaviour of the drug under different heating programmes. Doxorubicin nanocrystals were found to be stable after heating up to 80 °C, but an irreversible thermal behaviour was observed after a prolonged heating at elevated temperature (2 h at 80 °C). The non-reversibility could be related to the formation of a different ordered structure and enhanced by the slight leakage of the drug occurring after a prolonged heating.

1. Introduction

Liposomes are nano-sized lipid bilayer vesicles, widely used for drug delivery in the pharmaceutical field (Allen and Cullis, 2013; Samad et al., 2007). Liposomes have been successfully employed for the encapsulation of different drugs, including anticancer agents (Yingchoncharoen et al., 2016; Koudelka and Turánek, 2012). Due to their pharmacokinetics advantages, the second generation of liposomes (also called stealth liposomes), which are made up of a mixture of phospholipids and polymer-functionalized phospholipids, are the preferred for final dosage form formulations (Sen and Mandal, 2013). Indeed, the incorporation of polyethylene glycol (PEG) modified phospholipids in the vesicles prolongs their circulation time into the body, allowing passive targeting to inflamed and tumor sites (Sen and Mandal, 2013; Gabizon et al., 2003). Some PEGylated liposomal formulations have been approved for clinical use and many others are under investigation for cancer therapy (Shah et al., 2017). Doxil®/Caelyx (doxorubicin hydrochloride) is an example of a marketed liposomal formulation containing the anticancer drug doxorubicin hydrochloride (Barenholz, 2012). These stealth liposomes, composed of soy phosphatidylcholine, cholesterol and PEGylated phosphoethanolamine (mPEG-DSPE), have an average size of 80–90 nm and a stable loading of doxorubicin as sulphate salt after a remote loading via a transmembrane ammonium sulfate gradient (Wibroe et al., 2016). The mPEG-DSPE molecules, being incorporated during the preparation of the bilayer, extend the PEG chains from the liposome surface to the liposomes external environment and in the inner cavity as well (Schilt et al., 2016). When loaded in the liposomes, doxorubicin sulfate originates crystalline fibrillar supramolecular structures, which have been evidenced by x-rays scattering techniques and electron microscopy (Li et al., 1998). Recently, a further confirmation of the presence of nanocrystals of doxorubicin sulfate inside the liposomal formulation of Doxil® was given by high-sensitivity calorimetry (Wei et al., 2016). This technique has evidenced the presence of a narrow endothermic transition around 70 °C related to the melting of doxorubicin sulfate nanocrystals. This transition was found to be high cooperative and favoured by the restricted volume of the intra-liposomal environment. Moreover, in this work, using the calorimetric analyses, the reversibility

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of doxorubicin sulphate nanocrystals endothermic transition after thermal cycle (one cycled 15°–90°–15 °C heating/cooling scans) was demonstrated. The authors proposed the high sensitivity calorimetry as a versatile technique to investigate the thermal behaviour of complex stealth liposomal membrane and the physical state of the encapsulated drug. In addition, in the same paper, the physical and chemical stabilities of Doxil® liposomal formulation after two heating cycles between 15 °C and 90 °C was investigated (Wei et al., 2016).

The associated use of thermoresponsive liposomes formulation and the thermal ablation medical practice has revitalized the importance of investigating the thermal behaviour of liposomal formulation versus non temperature sensitive formulation (Rossmann et al., 2017). The recent liposomal formulations (lyso-thermosensitive liposomal doxorubicin LTLD, ThermoDox®), which respond to a heating stimulus (hyperthermia), are able to release around 80% of the encapsulated drug in 20 s at 42 °C and to deliver to the tumour a large amount of the therapeutic compared to the standard formulation (Dou et al., 2017; Landon et al., 2011).

In this paper, a detailed investigation on the thermal behaviour of doxorubicin-loaded liposomes was performed in comparison to the unloaded liposomal formulation (placebo). Data from a conventional thermal analysis technique as microcalorimetry (mDSC), were compared to those obtained from alternative methodologies as high-resolution ultrasonic spectroscopy (HR-US) and dynamic light scattering (counts analysis). Particularly, unloaded liposomes and doxorubicin-loaded liposomes were subjected to different thermal cycles in order to investigate deeply the behaviour over temperatures of the liposomal phospholipid membrane and the reversibility of the melting of the entrapped doxorubicin sulfate nanocrystals.

2. Materials and methods

2.1. Materials

Doxil® vials (loaded liposomes) and placebo (unloaded liposomes) have been supplied by Janssen Pharmaceutical Company of Johnson & Johnson. Doxil® is a liposomal formulation of doxorubicin (2 mg/mL) composed of a mixture of hydrogenated soy phospholipids, cholesterol and methyl-diasteroyl-phosphoethanolamine-polyethylenglicole 2 kDa (DSPE-PEG) sodium salt at the weight ratio 3:1:1. Doxil® and placebo are liposomal dispersions in 9.4% w/w sucrose (as main component) aqueous solution. The unloaded liposomes have the same qualitative and quantitative composition of the drug loaded formulation.

2.2. microDSC

Calorimetric studies were carried out using a microDSC III (Setaram, France). 0.750 g of the sample (placebo or loaded liposomes) and an equal amount of 9.4% sucrose solution as reference were filled in the a Hallostey calorimetric cells and equilibrated at 5 °C for 20 min before starting the analysis. Sample and reference were subjected to different thermal programmes. A first programme consists of two consecutive heating and cooling ramp from 5° to 80 °C at 1 °C/min. The second programme differs from the previous as the sample was left at 80° for two hours after the first heating ramp and before starting the subsequent cooling down ramp. The temperature (Tm,°C) and enthalpy (ΔH, J/g of solution) were calculated from the peak and the area of the transition, respectively, using the software of the instrument (Setsoft2000, Setaram) through the tangent method. Moreover, the thermodynamic parameters of loaded liposomes (Tm and $\Delta T_{1/2}$ as the temperature interval at half height of endotherm) were also calculated after a peak deconvolution performed using a multi-peaks non-linear modelling by fitting the thermograms with a Gaussian function (OriginPro8 software). For the reversibility study of thermal transitions, different aliquots of the sample (loaded liposomes), previously subjected to the thermal programme involving an isotherm for 2 h at 80 °C (as reported above), were sealed in tight-closed glass vials and stored at 4 °C or at room temperature (between 20°–25 °C). Different aliquots for each timepoints were analysed from 5 °C to 80 °C at 1 °C/min after 1 day, 3 days and 7 days of storage time. All measurements were performed in triplicates.

2.3. High-sensitive ultrasound spectroscopy

Ultrasonic attenuation and ultrasonic velocity of placebo and loaded liposomes were monitored as a function of temperature using a HR-US 102 high resolution spectrometer (Ultrasonic Scientific, Ireland) at the frequency of 5.4 MHz, preliminarily determined by a broad amplitude frequency scan. Around 2 mL of samples and reference (9.4% sucrose solution in water) were filled in the ultrasonic cells and left at 5 °C for at least 20 min for temperature equilibration and, then, subject to the same thermal programmes as described in mDSC analyses section. Temperature was controlled using a HAAKE C25P thermostat. Ultrasonic attenuation and sound speed are reported as differential values, obtained by subtracting the contribution of the reference from the value recorded in the sample cell. Sample transitions were calculated by the peak value from the attenuation profile or from the fist-derivate of the signal in the case of sound speed (Perinelli et al., 2013). All measurements were performed in triplicate.

2.4. Dynamic light scattering (DLS)

DLS analyses were performed using a Malvern Zetasizer nanoS (Malvern instrument Worcestershire, UK), detecting the scattered light at 173°. The scattered intensity (counts) of placebo and loaded liposomes, at a fixed position (4.65) and attenuation (10), were recorded in the range of temperature 5°–80 °C with a temperature step of 1 °C. The transition temperature was calculated from the Boltzmann regression curve as reported by Michel et al. (2006):

$$y = \frac{A_1 - A_2}{1 + e^{x - x_0/\Delta x}} + A_2 \quad (1)$$

Where A_1 and A_2 the plateaux of the curve, X_0 is the x value of the curve at the half distance between the plateau and Δx is the width of the slope.

For size analysis, measurements were performed using a viscosity value for the dispersant phase of 1.2994 cP at 20 °C. The hydrodynamic diameter of liposomes was calculated before and after each thermal programme and expressed as Z-average value by fitting the autocorrelation function with the “Cumulant model”. Particles size distribution was expressed as polydispersity index (PDI) and as percentile (X_{10}, X_{50} and X_{90}). Before each analysis, samples were equilibrated for 180 s at the operating temperature. Analyses were performed in triplicate.

2.5. UV and fluorescence analysis

The absorbance spectra of different aliquots of loaded liposomes were collected through a UV-spectrophotometer (UV-1800, Shimadzu). Loaded liposomes samples (diluted 1:40 in 9.4% sucrose solution) were analysed untreated or after thermal treatments as for mDSC and HR-US analyses. Not diluted untreated or after thermal treatments samples (0.5 mL) were also dialysed (Spetra/Por® Dialysis membrane MW cut-off 6–8000 Da) in 80 mL of 9.4% sucrose solution under stirring for 1 h. Previous dialyses were performed to ascertain no detectable release of the drug from the untreated loaded liposomes after up to 1 h. The dialysis medium and samples after dialysis were, then, collected and analysed through UV spectroscopy. The amount of doxorubicin released from the formulation was determined using a calibration curve, built up by measuring the absorbance of standard concentrations of doxorubicin hydrochloride at 485 nm (Supplementary Fig. SF1). Fluorescence was