Journal of Pharmaceutical and Biomedical Analysis 78–79 (2013) 118–122

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

Physicochemical characterization of liposomes after ultrasound exposure – Mechanisms of drug release

Tove J. Evjen[a,*], Stefan Hupfeld[b], Sabine Barnert[c], Sigrid Fossheim[b], Rolf Schubert[c], Martin Brandl[d]

[a] Epitarget AS, Oslo, Norway
[b] Clavis Pharma ASA, Oslo, Norway
[c] University of Freiburg, Department of Pharmaceutical Technology and Biopharmacy, Freiburg, Germany
[d] University of Southern Denmark, Department of Physics, Chemistry and Pharmacy, Odense, Denmark

ARTICLE INFO

Article history:
Received 22 October 2012
Received in revised form 26 January 2013
Accepted 28 January 2013
Available online 15 February 2013

Keywords:
Liposome
Ultrasound
Drug release
Release mechanism
Asymmetric flow field-flow fractionation

ABSTRACT

Ultrasound is investigated as a novel drug delivery tool within cancer therapy. Non-thermal ultrasound treatment of solid tumours post i.v.-injection of drug-carrying liposomes may induce local drug release from the carrier followed by enhanced intracellular drug uptake. Recently, ultrasound-mediated drug release of liposomes (sonosensitivity) was shown to strongly depend on liposome membrane composition. In the current study the ultrasound-mediated drug release mechanism of liposomes was investigated. The results showed that differences in ultrasound drug release kinetics obtained for different liposomal compositions were caused by distinctive release mechanisms of the carriers. Two types of liposomes composed of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) and hydrogenated soy L-α-phosphatidylcholine (HSPC) as main lipids, respectively, were recently shown to vary in sonosensitivity. Here, these liposomes were analyzed prior to and after a given ultrasound-exposure for their mean size, size distribution and morphology. Cryo-transmission electron microscopy, dynamic light scattering and asymmetric flow field-flow fractionation in combination with multi-angle light scattering revealed a significant change in mean size, size distribution and morphology of DOPE-based liposomes after ultrasound, pointing to an irreversible disruption of the vesicles and concomitant drug release. In contrast, the HSPC-based liposomes remained unchanged in size and structure after ultrasound application, indicating pore-mediated release mechanisms. The results show that the release mechanisms and interactions between ultrasound and liposomes depend on the liposome membrane-composition, explaining their sonosensitive properties.

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1. Introduction

Non-thermal low frequency ultrasound is currently investigated as a novel drug delivery tool within oncology. (For a review see [1]). Ultrasound may improve drug delivery to cancer cells via administration of drug containing carriers [2,3]. Ultrasound treatment of tumour tissue upon accumulation of liposomes may induce local drug release from the liposome followed by increased uptake into tumour cells [1]. Although several animal studies have shown a therapeutic benefit of this strategy [2–4], the underlying mechanisms of liposomal drug release and interactions between ultrasound and phospholipid-membranes are not well understood [5].

It is assumed, though, that acoustic cavitation represents the primary physical mechanism behind non-thermal ultrasound-mediated drug delivery (For a review and references therein see Pitt et al. [5]). Cavitation is defined as the growth, oscillation and collapse of small, stabilized gas bubbles under the influence of varying pressure field of a sound wave in a fluid medium [6]. The formation and collapse of micro-bubbles preferentially occurs near interfaces such as the surface of cells or liposomes surface, which then is believed to induce diffusion of macromolecules across the membranes [6]. Several studies have demonstrated, however, that ultrasound-mediated drug release or sonosensitivity of liposomes widely varies with their membrane composition [7–10]. Recently, Evjen et al. found that non-bilayer forming lipids with large hydrophobic cross section as compared to the polar headgroup (packing parameter (PP) >1) significantly promoted liposome sonosensitivity [9,10]. Inclusion of dioleoylphosphatidylethanolamine (DOPE) within liposome membranes comprising phosphatidylcholines (PCs), PEGylated

Abbreviations: AF4/MALS, asymmetric flow field-flow fractionation/multi-angle light scattering; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; HSPC, hydrogenated soy L-α-phosphatidylcholine; 1,, 2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-(methoxy(polyethylene glycol)-2000) (DSPE-PEG 2000).

* Corresponding author at: Epitarget AS, PO Box 7159 Majorstuen, 0307 Oslo, Norway. Tel.: +47 23201200; fax: +47 23201249.
E-mail address: tove.julie@gmail.com (T.J. Evjen).

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http://dx.doi.org/10.1016/j.jpba.2013.01.043

distearoylphosphatidylethanoamine (DSPE-PEG 2000) and cholesterol significantly enhanced the release rate of the anthracycline doxorubicin (DXR) on exposure to 40 kHz ultrasound [9]. Preliminary cryo-transmission electron microscopy (Cryo-TEM) data indicated a disruption of the liposomal membrane on ultrasound stimulation, which correlates with the rapid drug release [9].

In the current study, the mechanisms of ultrasound-mediated drug release from liposomes were further investigated. The aim was to examine whether the observed differences in ultrasound-mediated drug release kinetics from different liposomes might be attributed to membrane specific release mechanisms or rather liposome stability per se. Two liposome formulations (DOPE- and HSPC-based liposomes) were investigated after ultrasound-exposure that corresponded to a similar DXR release extent. Liposome size, size distribution and morphology of both formulations were characterized before and after ultrasound using Cryo-TEM, photon correlation spectroscopy (PCS) and asymmetric flow field-flow fractionation/multi-angle light scattering (AF4/MALS).

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*- (methoxy (polyethylene glycol)-2000) (DSPE-PEG 2000) were purchased from Genzyme Pharmaceuticals, Liestal, Switzerland. Cholesterol, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), ammonium sulphate, sodium azide, Triton®X-100 solution and sucrose were obtained from Sigma Aldrich, Oslo, Norway. Doxorubicin hydrochloride (DXR) was purchased from Nycomed, Asker, Norway. DXR-containing liposomes, Caelyx® (herein termed HSPC-based liposomes) composed of hydrogenated soy phosphatidylcholine, DSPE-PEG 2000 and Chol (57:5:38 mol %) was supplied from the pharmacy at the Norwegian Radium Hospital, Oslo, Norway (European distributor Schering-Plough). The carrier liquid used for AF4/MALS experiments was 10 mM sodium nitrate (Merck KGaA, Darmstadt, Germany). The solution was prepared using ultrapure water (prepared by Milli-Q® advantage A10®) and was filtered through a membrane filter with a pore size of 0.22 μm prior to use.

2.2. Liposome preparation

DOPE-based liposomes with the lipid composition DOPE:DSPC:DSPE-PEG 2000:cholesterol (62:10:8:20 mol%) were prepared by the thin film hydration and sequential extrusion method as previously described [9]. The liposomes were remote loaded with DXR using a transmembrane ammonium sulphate gradient obtained by dialysis as previously described [9]. After liposome preparation 0.01% (w/v) sodium azide was added as a preservative.

2.3. Ultrasound exposure

Ultrasound-exposure was conducted using a 40 kHz ultrasound transducer (VC 750, Sonic and Materials, Inc, Newtown, CT, USA) with a 19 mm diameter non-focused probe as previously described [11]. The liposome-dispersions were diluted 1:10 (v/v) ratio with 0.22 μm-filtered sucrose/HEPES solution prior to ultrasound-exposure. 10 ml of the diluted liposome solutions were immersed into a glass vial, and the ultrasound transducer was immersed directly into the liposome dispersions. A water bath was used to control the sample temperature around room temperature. The maximum temperature after 6 min of sonication was 27 °C, excluding the possibility of temperature-induced drug release. Ultrasound was run at a nominal intensity of 12 W/cm^2 in a continuous mode (100% duty cycle). The HSPC-based liposomes and DOPE-based liposomes were exposed to ultrasound for 6 min and 2 min resulting in approximately 63% release of DXR. DXR release from liposomes after ultrasound exposure was measured fluorometrically as previously described [10].

2.4. Cryo-TEM

Cryo-TEM investigations were performed according to methods described by Rank et al. [12], using a LEO 912 OMEGA electron microscope (Zeiss, Oberkochen, Germany) operating at 120 kV. Ultrasound-treated liposome dispersions were diluted 1:10 (v/v) with 0.22 μm-filtered sucrose/HEPES solution prior to ultrasound. The untreated liposome dispersions were not diluted prior to analysis. Sample preparation and investigations were performed as previously described [9].

2.5. Photon correlation spectroscopy

The mean intensity-weighted hydrodynamic diameter was determined by PCS (Nanosizer, Malvern Instruments, Malvern, UK) before and after ultrasound treatment. Prior to measurements the liposome dispersions were diluted 1:200 (v/v) with 0.22 μm-filtered sucrose/HEPES buffer. The measurements were performed at 23 °C and at a scattering angle of 90°. The width of the particle size distribution was expressed by the polydispersity index (PI). Measurements were performed in triplicates.

2.6. Asymmetric flow field-flow fractionation

The AF4/MALS experiments were performed according to methods described by Hupfeld et al. [13]. The AF4 system consisted of a HPLC pump (G1314A Iso pump, 1200 series Agilent Technologies, Santa Clara, CA, USA) connected to an Eclipse 3+ separation system (Wyatt Technology Europe GmbH, Dernbach, Germany), an 18-angle static light-scattering detector (DAWN EOS, Wyatt Technology Corp., Santa Barbara, CA, USA), a variable wavelength UV–vis detector (G1314A VWD detector, Agilent technologies) and a differential refractive index detector (dRI) (Optilab rEX 949-rEX, Wyatt Technology Corp.). The AF4 channel had a tip-to-top length of 14.5 cm and a channel height of 250 μm fitted with a semi-permeable membrane consisting of 10 kDa regenerated cellulose. Particle size calculations were performed using the coated sphere model in Astra Version 6 software (Wyatt Technology Corp). Relaxation of the sample consisted of injection and focusing and was performed from 0 to 7 min at a focusing flow of 1.5 ml/min. The sample injection volume was 5 μl. A channel flow of 1.0 ml/min was used during the elution step. A cross flow gradient of 1.0–0.1 ml/min was applied for 15 min, followed by an isocratic cross flow of 0.1 ml/min for 25 min. Finally, the system was flushed for 15 min without cross flow. All experiments were performed in triplicates.

3. Results and discussion

Sonosensitivity of liposomes has previously shown to strongly vary with their lipid membrane composition [7–10]. Inclusion of DOPE into liposome membranes was found to significantly enhance sonosensitivity [9]. Faster drug release kinetics for DOPE-based liposomes compared to HSPC-based liposomes was also observed in the current study. Approximately 63% of the entrapped DXR was

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