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Short communication

The use of asymmetrical flow field-flow fractionation with on-line detection in the study of drug retention within liposomal nanocarriers and drug transfer kinetics

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ABSTRACT

Due to their solubilizing capabilities, liposomes (phospholipid vesicles) are suited for designing formulations for intravenous administration of drug compounds which are poorly water-soluble. Despite the good in-vitro stability of such formulations with minimal drug leakage, upon i.v. injection there is a risk of premature drug loss due to drug transfer to plasma proteins and cell membranes. Here we report on the refinement of a recently introduced simple in vitro predictive tool by Hinna and colleagues in 2014, which brings small drug loaded (donor) liposomes in contact with large acceptor liposomes, the latter serving as a model mimicking biological sinks in the body. The donor- and acceptor-liposomes were subsequently separated using asymmetrical flow field-flow fractionation (AF4), during which the sample is exposed to a large volume of eluent which corresponds to a dilution factor of approximately 600. The model drug content in the donor- and acceptor fraction was quantified by on-line UV/VIS extinction measurements with correction for turbidity and by off-line HPLC measurements of collected fractions. The refined method allowed for (near) baseline separation of donor and acceptor vesicles as well as reliable quantification of the drug content not only of the donor- but now also of the acceptor-liposomes due to their improved size-homogeneity, colloidal stability and reduced turbidity. This improvement over the previously reported approach allowed for simultaneous quantification of both drug transfer and drug release to the aqueous phase. By sampling at specific incubation times, the release and transfer kinetics of the model compound *p*-THPP *(5,10,15,20-tetrakis(4-hydroxyphenyl)21H,23H-porphine)* was determined. *p*-THPP is structurally closely related to the photosensitizer temoporfin, which is in clinical use and under evaluation in liposomal formulations. The transfer of *p*-THPP to the acceptor vesicles followed 1st order kinetics with a half-life of approximately 300 min. As expected, equilibrium distribution between donor- and acceptor vesicles was proportional to the lipid mass ratio. An initial rapid transfer of *p*-THPP was found (~5%) and investigated further by determining the extent of transfer between donor and acceptor during separation. The donor- and acceptor phase were found to be separated within few minutes and only minor (≤2%) transfer could be detected within the AF4 channel under the conditions applied for fractionation. These results demonstrates the potential of our AF4 based method as an in vitro tool to determine retention properties of lipophilic compounds within liposomal carriers in particular, but also within a variety of nano-particulate carriers provided that they exhibit a sufficient size difference compared to the applied colloidal acceptor phase.

1. Introduction

Phospholipids are popular excipients for designing parenteral formulations for amphiphilic and lipophilic drugs due to their good biocompatibility and lower toxicity compared to synthetic solubilizers [1]. Phospholipid vesicles (liposomes), which contain the drug incorporated in the bilayer, typically show high drug loading efficiencies and excellent physical stability in terms of drug leakage due to the inherent affinity of the drug for the lipid bilayer. In contrast to water-soluble drugs, which typically are released once there occurs a drug concentration gradient across the bilayer,

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poorly water-soluble drugs tend to remain associated with the liposome bilayers even upon extensive dilution [2].

In dual-labelling studies it could be shown that drug substance and liposomes upon i.v. injection are subject to different clearance and pharmacokinetics indicating premature drug-loss: Amphotericin, when injected in its liposomal formulation, has been reported to be cleared much more rapidly from circulation than the liposomes [3]. For liposomal temoporfin, a fraction of the drug appeared to be released from the liposomes prior to being eliminated from the blood [4,5]. Such loss of drug from the liposomal carrier is postulated to be due to transfer of the drug to various biological sinks such as plasma proteins (e.g. albumin, HDL, LDL) and cell membranes.

Such incident may not matter as long as the intention with a liposomal formulation is to achieve solubilization of the drug rather than controlled release or drug targeting. For targeting of tumors or inflamed tissues by colloidal systems via the EPR effect, however, the lipophilic drug should stay associated with the carrier during transport in the blood stream until accumulation in the target tissue is accomplished. If not, controlled drug release and/or targeted delivery of drugs to specific organs or tissues by such liposomal formulations may be impaired, depending on the rate and extent of premature drug-loss. It appears thus important to establish a simple predictive in vitro assay to determine the drug release- and transfer kinetics from such nanocarriers and a variety of in vitro approaches has been suggested in recent years [6–14]. For a critical review of the various approaches to determine nanoparticulate retention, please see [15].

2. Materials and methods

2.1. Materials

A commercial blend of egg phospholipid (Lipoid E80S) was kindly provided by Lipoid GmbH (Ludwigshafen, Germany). *p*-THPP *(5,10,15,20-tetrakis(4-hydroxyphenyl) 21H,23H-porphine)*, trimethylphosphate, methanol, chloroform, glycerol, Triton® X-100, and sodium azide were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and TRIS *(Tris(hydroxymethyl) aminomethane)* from Merck KGaA (Darmstadt, Germany). All solvents were HPLC grade purity. Purified water was obtained by a laboratory water purification system (Milli-Q® Advantage A10® system, Merck-Millipore, Merck KgaA (Darmstadt, Germany)).

2.2. Composition preparation and characterization of the liposomes

Small liposomes composed of 5% (m/V) phospholipid (E80S) with or without *p*-THPP, dispersed in isotonic and preserved TRIS buffer (2.45% glycerol and 0.02% sodium azide) were prepared as described earlier [16], Large acceptor liposomes were prepared using a modified protocol described recently [17].

The average particle size (hydrodynamic diameter) was determined by dynamic light scattering as previously described [16,18].

The lipid content of the non-loaded small liposomes and the centrifuged acceptor liposomes was determined by an enzymatic assay as described in [19] and together with model drug-loaded liposomes also by ^{31}P NMR as described [20]. Samples for ^{31}P NMR contained approximately 5 mg/mL E80S dispersed in isotonic TRIS buffer containing 10% (v/v) Triton-X®-100, 10% (v/v) D_2O and 1 mg/mL of the internal standard trimethylphoshpate.

2.3. Asymmetrical flow field-flow fractionation (AF4)

AF4-MALLS instrument setup and procedures were as described earlier [18,21–24]. For the transfer studies, diluted liposome dispersions were injected into the channel (180 μg injected sample mass). Experimental parameters are summarized in Table 1.

2.4. Off-line quantification of model compounds

Triplicate off-line UV/VIS absorbance measurements of liposomal samples were carried out in methanolic solutions (≥1:10 dilution) using a Waters 2695HPLC with UV detection (Waters 2487 Dual λ Absorbance Detector, Waters GmbH, Eschborn, Germany). Separation was obtained using an Acclaim® 120 (C18, 3 μm particle size, 120 Å, 4.6 mm × 150 mm) column (Dionex, Hvidovre Denmark). Isocratic HPLC measurements were carried out at 418 nm with methanol as eluent with a detector flow of 1 mL/min and a sample injection volume of 100 μL. An appropriate calibration curve was established, with $R \geq 0.99974$ and LOD and LOQ of 1.8 and 5.5 ng/mL respectively based on the standard deviation of the response and slope according to ICH Q2B guideline [25]. The software used was Chromeleon 6.80 (Dionex, Hvidovre, Denmark).

2.5. On-line quantification of p-THPP

The content of the model drug *p*-THPP in the donor- and acceptor fraction was quantified from on-line measurements as described earlier [21], by correcting the measured UV/VIS extinction for turbidity using non-loaded control samples.

2.6. Transfer studies

To investigate the applicability of on-line UV/VIS measurements to determine *p*-THPP content in donor- and acceptor liposomes during incubation and the kinetics of transfer, small donor liposomes (NL-DL and *p*-THPP-DL) and large acceptor liposomes (AL) were mixed in a 1:0.8 mass ratio of DL-to-AL with a total lipid concentration of 45 mg/mL. The experiments were carried out in

Table 1
AF4 method flow parameters.

AF4 Method	aCh-I	aCh-II	aCh-III	Double injection	Incubation
Detector flow (V_D)	1 mL/min	1 mL/min	1 mL/min	1 mL/min	1 mL/min
Focus flow (V_F)	2 mL/min	2 mL/min	2 mL/min	1 mL/min	0.1 mL/min
Injection flow (V_{INJ})	0.2 mL/min	0.2 mL/min	0.2 mL/min	0.2 mL/min	0.2 mL/min
Mode	Crossflow gradient (mL/min)				
Focus	Focus flow for 11 min	Focus flow for 11 min	Focus flow For 5 min	Focus flow 5 min	Focusflow 10, 30 or 60 min
Elution	2–0.5 over 5 min	2–0.5 over 5 min	2–0.5 over 5 min		
Elution	0.5–0[a] over 30 min	0.5–0.07 over 25 min	0.5–0.07 over 25 min		
Elution	0 for 10 min	0.07 for 15 min	0.07 for 15 min		
Elution	0 for 10 min	0 for 10 min	0 for 10 min		
Method length	66 min	66 min	60 min		

[a] Crossflow drops to 0 mL/min around 42–43 min.