

Quantitative *In Vitro* Assessment of Liposome Stability and Drug Transfer Employing Asymmetrical Flow Field-Flow Fractionation (AF4)

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ABSTRACT

Purpose In the present study we introduce an efficient approach for a size-based separation of liposomes from plasma proteins employing AF4. We investigated vesicle stability and release behavior of the strongly lipophilic drug temoporfin from liposomes in human plasma for various incubation times at 37°C.

Methods We used the radioactive tracer cholesteryl oleyl ether (COE) or dipalmitoyl-phosphocholine (DPPC) as lipid markers and ¹⁴C-labeled temoporfin. First, both lipid labels were examined for their suitability as liposome markers. Furthermore, the influence of plasma origin on liposome stability and drug transfer was investigated. The effect of membrane fluidity and PEGylation on vesicle stability and drug release characteristics was also analyzed.

Results Surprisingly, we observed an enzymatic transfer of ³H-COE to lipoproteins due to the cholesterol ester transfer protein (CETP) in human plasma in dependence on membrane rigidity and were able to inhibit this transfer by plasma preincubation with the CETP inhibitor torcetrapib. This effect was not seen when liposomes were incubated in rat

plasma. DPPC labels suffered from hydrolysis effects during preparation and/or storage. Fluid liposomes were less stable in human plasma than their PEGylated analogues or a rigid formulation. In contrast, the transfer of the incorporated drug to lipoproteins was higher for the rigid formulations.

Conclusions The observed effects render COE-labels questionable for *in vivo* studies using CETP-rich species. Here, choline labelled ¹⁴C-DPPC was found to be the most promising alternative. Bilayer composition has a high influence on stability and drug release of a liposomal formulation in human plasma.

KEY WORDS asymmetrical flow field-flow fractionation · CETP · drug transfer · liposomes · plasma proteins

ABBREVIATIONS

AF4	Asymmetrical flow field-flow fractionation
CETP	Cholesteryl ester transfer protein
COE	Cholesteryl oleyl ether
DLS	Dynamic light scattering
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
DPPG	1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol, sodium salt
EPC	L- α -phosphatidylcholine (Egg Chicken)
EPG	L- α -phosphatidylglycerol, sodium salt (Egg, Chicken)
EPR	Enhanced permeation and retention
HDL	High density lipoprotein
LDL	Low density lipoprotein
MALLS	Multi angle laser-light scattering
mTHPC	Temoporfin
PCS	Photon correlation spectroscopy
RES	Reticuloendothelial system
VLDL	Very low density lipoprotein

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INTRODUCTION

Among all drug molecules entering the market or being promising candidates in the research pipelines of pharmaceutical companies the percentage of highly hydrophobic, water-insoluble drugs is increasing. Hence, solubilisation of hydrophobic substances for mainly intravenous application is a formulation task continuously gaining importance. As the number of potential solvents is limited by toxicity aspects modern and complex solutions like nanosized drug carriers come into focus of present research activities (1).

Liposomes are one prominent example of nanosized carriers and have been investigated in a plethora of publications since their discovery by Alec Bangham in 1961 (2,3). As their main components phospholipids are the elementary part of human membranes, liposomes are regarded as biocompatible and safe (4). Due to their amphiphilic nature, phospholipids spontaneously form concentric vesicles and liposomes in aqueous environment. Within the lipophilic region of these bilayers hydrophobic substances can be incorporated. By the choice of lipids, the employment of membrane additives or surface modifications it is possible to modify the release profile of the drug formulation (5). Furthermore, liposomes can reach the site of action passively by virtue of the enhanced permeability and retention (EPR) effect or actively by surface modification with antibodies. Thus, the main applications of liposomes are either drug solubilisation or targeting purposes.

If drug solubilisation is the main formulation aim, high liposome stability and stable drug incorporation is not required. Here, fast liposome destruction or uptake by the reticuloendothelial system (RES) and a rapid drug transfer to lipophilic binding domains such as red blood cell membranes, lipoproteins or albumin may be desired. Small liposomes (increased specific surface area) with a liquid-crystalline membrane state ("fluid liposomes") are a possible approach to overcome this issue (6). In contrast, a more ambitious aim is the use of liposomal carriers for drug targeting purposes. In this case, highly stable, long circulating, non- or late-disintegrating vesicles are needed and the drug should be firmly incorporated within the lipophilic core of the liposomal bilayer in order to reach the site of action. Addition of cholesterol or PEGylated lipids to the bilayer or the usage of phospholipids with a phase transition above the body temperature ("rigid liposomes") are beneficial for this intention (7–9). However, because of a missing protective barrier between drug molecules and potential acceptor sites, collision and diffusion mechanisms can lead to an early enhanced release of the drug out of the liposomal bilayer. Lipoproteins are quite abundant in blood and come immediately into contact with liposomes after their intravenous injection. Consequently, recent pharmacokinetic studies have shown that e.g. the lipophilic photosensitizer Temoporfin (mTHPC) is rapidly released from rigid liposomes and transferred to lipophilic acceptor domains

(5,10,11). Hence, simultaneous investigations on vesicle stability and drug transfer in presence of human plasma *in vitro* are important tools for the understanding of liposomal interactions with physiological fluids.

In order to investigate liposome – plasma protein interactions as a function of time, both species need to be separated again after an incubation period. In the past, separation methods based on size exclusion chromatography (SEC) (7,11–13) or density gradient ultracentrifugation (8) seemed to be promising approaches to measure these interactions.

Asymmetrical flow field-flow fractionation (AF4) is another analytical tool for the separation of complex samples over a large colloidal size range without the need of a stationary phase. Hence, non-specific interactions and shear forces are reduced to a minimum ensuring a gentle separation. In AF4, particles are separated according to their hydrodynamic size with the smallest particles eluting first. By adjusting various parameters a well-defined separation can be achieved within an appropriate time range (14). AF4 already proved its suitability for the separation and characterization of human plasma within a short time (15,16). It has also been proven as a successful tool for drug-protein-interactions (17). Likewise, there are several publications focusing in depth on the characterization of liposomal formulations (18–20). Moreover, there has been a recent study about investigations on drug transfer of the lipophilic photosensitizer p-THPP between liposomes of different size ranges via on-line detection (21).

The aim of this study was to show the feasibility of AF4 for a comparably fast and high-resolving separation of an incubated plasma-liposome sample in respect of time, reproducibility and comparability with established methods. Furthermore, we were focusing on principal aspects of the experimental design, which may influence such experiments extraordinarily. Therefore, first experiments were performed employing a well-known liposomal formulation. mTHPC was incorporated either in fluid EPC/EPG or more rigid DPPC/DPPG liposomes, which have already been intensively investigated in the past (5,7,10,11). For analytical purposes, liposomes were labelled by a radioactive lipid label indicating liposomal stability and radioactive labelled drug was used as a tracer for the drug transfer. In total, the suitability of four lipid labels was compared and difficulties with label hydrolysis and enzymatic exchange had to be investigated further. Furthermore, the influence of the plasma origin and lipid composition on liposome stability and drug transfer was examined.

MATERIAL AND METHODS

Materials

The phospholipids L- α -phosphatidylcholine (egg, chicken; EPC) as well as L- α -phosphatidylglycerol, sodium salt (egg,

chicken; EPG, Na) were kindly provided by Lipoid GmbH (Ludwigshafen, Germany) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol, sodium salt (DPPG, Na) as well as 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt) (mPEG₂₀₀₀-DSPE) by Genzyme (Liestal, Switzerland), respectively. Temoporfin (mTHPC; 5,10,15,20-tetrakis (3-hydroxyphenyl)porphyrine) and ¹⁴C-mTHPC were supplied by biolitec Research GmbH (Jena, Germany). The radioactive lipid labels ³H-cholesteryl oleyl ether (³H-COE), ³H-DPPC and both ¹⁴C-DPPC were all purchased from Biotrend Chemicals (Cologne, Germany). Lipoproteins, low density (LDL, human) and high density (HDL, human) as well as albumin (human), Sudan Black B, Methanol (HiPerSolv CHROMANORM® HPLC gradient grade > 99%) and ethanol (AnalaR Normapur®, ≥ 99%, Ph. Eur. for synthesis) were obtained from VWR International GmbH (Darmstadt, Germany). Chloroform (Rotisolv® HPLC), dimethyl sulfoxide (DMSO, ROTIPURAN®, (≥99.8% p.a.), sodium chloride (≥99.8%), TRIS (Pufferan®, ≥99.9%, p. a.), sodium azide (≥99%, p. a.), hydrochloric acid (2 N), and Rotiszint® Eco plus, a ready-to-use scintillation cocktail, were all obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). α-D (+)-glucose anhydrous was purchased from Roquette GmbH (Frankfurt, Germany) and torcetrapib (≥98%, HPLC grade), a cholesteryl ester transfer protein (CETP) inhibitor, from Sigma-Aldrich (St. Louis, MO, USA), respectively.

Ultrapure water (RIOs™ 8, Milli-Q^R Advantage A10^R System, Merck Millipore, Merck KGaA, Darmstadt, Germany) was employed for preparation purposes and purified water (Millipore Direct Q™, Merck Millipore) for size determination measurements, respectively.

Liposome Composition and Preparation

Table I summarizes all prepared and investigated formulations. Principally, fluid EPC/EPG liposomes (9:1 (*w/w*), 20 mg/ml) were compared with DPPC/DPPG liposomes (9:1 (*w/w*), 20 mg/ml) representing a formulation with increased bilayer rigidity at 37°C. The hydrophobic model drug temoporfin was incorporated into both liposomal membranes at a concentration of 8 mol% relative to phospholipids. Traces of ¹⁴C-temoporfin (40 μCi/ml liposome suspension) were used as a representative marker for drug transfer. As liposomal membrane labels ³H-COE, ³H-DPPC or ¹⁴C-DPPC were used. The ¹⁴C-atom was either located at a fatty acid or the choline methyl group. All formulations were prepared in 5% (*w/v*) glucose solution (sterile syringe filter, 0.2 μm pore size, VWR, Darmstadt, Germany).

All liposomes were prepared by using the thin lipid film hydration method followed by extrusion (22). In brief, lipids and temoporfin were dissolved in chloroform or ethanol,

respectively. Radioactive labels were already obtained as stock solution in toluol or ethanol. All stock solutions were mixed together in a specified ratio and film formation was performed by evaporating the organic solvents using a rotary evaporating system (Rota Vapor R-114 and Vacobox B-177, Büchi Labortechnik GmbH, Essen Germany) and drying the film for at least 2 hours. Rehydration of the dried lipid film was performed by adding glucose solution and gently shaking. After an equilibration time of at least 1 hour the resulting dispersion of multilamellar liposomes was extruded 41 times through polycarbonate membranes with a nominal pore size of 100 nm (Armatis, Mannheim, Germany) employing a hand extruder (LiposoFast Basic, Avestin Inc., Ottawa, Canada). The whole preparation process was carried out well above the phase transition temperature of the lipids and under light protection. The final formulations were stored at 4°C until they were used. PEGylated liposomes were purified by ultracentrifugation (Beckman XL80 equipped with a SW 55 Ti rotor, Beckman Coulter, Brea, CA, USA) at 200,000 g at 4°C for 3 h.

Photon Correlation Spectroscopy (PCS)

Size determination was carried out directly after liposome preparation employing dynamic light scattering measurements (Zetasizer NanoZS) in the backscattering mode (173°). Optimal measurement conditions were indicated by an attenuator index between 6 and 8 and achieved by sample dilution with 5% (*w/v*) glucose solution (sterile syringe filter, 0.2 μm pore size). The temperature was set to 25°C and a sample viscosity of 1.0600 cP (glucose 5% *w/w* at 25°C) was assumed (23). Measurements were either performed in the manual (four runs, each lasting 5 min) or in the automatic (four runs, each with twelve to 16 measurements) mode.

Plasma Preparation

Human blood was collected from five healthy volunteers (three male, two female) having fasted for 12 hours. Rat blood was obtained from male Wistar rats. The donated blood was centrifuged (Eppendorf 5804R, Eppendorf AG, Hamburg, Germany) at 4500 relative centrifuge force (rcf) for 10 min. Aliquots of the obtained plasma were frozen to -20°C and thawed on the day they were used. Aliquots were filtered through a 0.2 μm RC syringe filter (Rotilabo® Mini-Tip, Carl Roth, Karlsruhe, Germany). If not stated otherwise, plasma of one male donor was used and samples for investigations on transfer kinetics were prepared by mixing 1.5 μmol liposomal lipid with 500 μl plasma and diluted with buffer to a final volume of 1 ml (12). Incubation was performed at 37°C in a water bath under light protection and for defined time intervals ranging from 0.5 to 48 h. Afterwards, the sample was

Table 1 Liposome Composition for Method Establishment and Temoporfin Transfer Studies

No.	Formulation	³ H [μ Ci/ml]	¹⁴ C [μ Ci/ml]	z-Average [nm]	PDI
1	EPC/EPG (9:1 (w/w), 20 mg/ml) mTHPC (8 mol%)	COE 10 μ Ci/ml	DPPC ^a 5 μ Ci/ml	112 \pm 1	0.09 \pm 0.01
2	EPC/EPG (9:1 (w/w), 20 mg/ml) mTHPC (8 mol%)	COE 40 μ Ci/ml	mTHPC 40 μ Ci/ml	96 \pm 3	0.09 \pm 0.01
3	EPC/EPG (9:1 (w/w), 20 mg/ml) mPEG ₂₀₀₀ -DSPE (6 mol%) mTHPC (8 mol%)	COE 10 μ Ci/ml	DPPC ^a 5 μ Ci/ml	89 \pm 1	0.08 \pm 0.01
4	DPPC/DPPG (9:1 (w/w), 20 mg/ml) mTHPC (8 mol%)	COE 10 μ Ci/ml	DPPC ^a 5 μ Ci/ml	112 \pm 1	0.09 \pm 0.01
5	DPPC/DPPG (9:1 (w/w), 20 mg/ml) mTHPC (8 mol%)	DPPC ^a 4 μ Ci/ml	DPPC ^b 2 μ Ci/ml	102 \pm 1	0.09 \pm 0.03
6	DPPC/DPPG (9:1 (w/w), 20 mg/ml) mTHPC (8 mol%)	COE 40 μ Ci/ml	mTHPC 40 μ Ci/ml	85 \pm 2	0.07 \pm 0.01

^a Label is located at a fatty acid

^b Label is located at a choline methyl group

applied to the separation device as described below. Non-separated sample was kept as a reference.

Asymmetrical Flow Field-Flow Fractionation (AF4)

AF4 measurements were performed at 25°C on an AF2000 MT system (Postnova Analytics, Landsberg, Germany) coupled to a refractive index (RI, PN 3150), multi-angle laser light scattering (MALLS, PN3070), dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments, Herrenberg, Germany) and a variable wavelength (UV/Vis, PN3211) detector. The UV/Vis detector was set at a wavelength of 280 nm if not stated otherwise. Data were collected at intervals of 0.5 s and evaluated by the AF2000 Control software (version 1.2.0.19). For size analysis of the liposome fraction using the MALLS-detector, a sphere model was used to calculate the radius of gyration (R_g).

The separation channel was equipped with a trapezoidal spacer with an overall area of 31.6 cm² and a height of 500 μ m. A membrane of regenerated cellulose with a MWCO of 10 kDa served as accumulation wall. A buffer composition of 150 mM NaCl, 10 mM Tris (pH 7.4) and 0.03% (*w/v*) NaN₃ was used as carrier liquid and for sample dilutions.

Samples were eluted with a detector flow rate of 1 ml/min. The sample was injected via a loop injector with a volume of 20.31 μ l (specified by the vendor) into the channel during the focusing step with an injection flow rate of 0.2 ml/min, a cross-flow rate of 4 ml/min and a resulting focus-flow rate of 4.2 ml/min over 12 min. After a transition time of 1 min the cross-flow was kept constant at 4 ml/min for 18 min and then decreased exponentially within 30 min to 0.4 ml/min and within another 10 min to 0.01 ml/min. The cross-flow was kept constant at 0.01 ml/min for 15 min and at 0.00 ml/min for another 4 min to ensure complete sample elution. Samples

were collected for further analyses in 1 ml fractions between 18 and 82 min.

To evaluate whether system parameters (e. g. conditions during the elution or focus step) have an influence on drug transfer and to determine the experimental time point $t = 0$ as realistically as possible liposomes and human plasma were mixed when starting the separation by injecting them separately. Therefore, an equivalent amount of human plasma was injected into the AF4 channel as described above. Subsequently, the injection loop was opened again after 2 minutes and an equivalent amount of liposome formulation was injected. The sample was focused for 10 more minutes and separated as described above.

Lipoprotein Staining and Peak Identification

Identification of the peaks for albumin, HDL and LDL was achieved with the help of protein standards. According to average human lipoprotein levels (24) and albumin reference ranges (25), solutions with a albumin level of 5 g/dl, HDL cholesterol level of 40 mg/dl or a LDL cholesterol concentration of 140 mg/dl were prepared, diluted with buffer and separated as described above. The resulting elution profiles were compared with the profile obtained for human plasma.

Additionally, human plasma and the standard solutions were stained with Sudan Black B according to the following protocol (15): 250 μ l human plasma or a corresponding amount of protein standards were diluted with buffer to 500 μ l. Fifteen microliter of 1% (*m/v*) Sudan Black B in DMSO were added and the samples were stirred gently for 20 min. Samples were separated with AF4 as described before with the UV/Vis detector set at a wavelength of 600 nm.

Liposome – Plasma Protein Interactions: Suitability of Lipid Labels

For selected formulations the stability in plasmas obtained from five different people was investigated. Furthermore, the suitability of various lipid labels as a marker for liposome stability was examined (Table I). To evaluate the effect of hydrolysis of DPPC labels e.g. during liposome preparation, storage or incubation on their transfer behavior, stability of liposomes traced with two different DPPC labels was investigated after preparation and after 9 month storage.

In order to clarify whether the employed lipid label ^3H -COE is transferred by CETP or not, further incubation experiments were performed with some modifications. First, incubation temperature was changed to 4°C . Second, liposomes were incubated with isolated lipoproteins (pure HDL or LDL standard solutions as described above). Both standards were mixed with liposomes and diluted with buffer separately. Samples were incubated at 37°C for 2 hours in a water bath and subsequently separated by AF4. Third, rat plasma was used instead of human plasma.

CETP Inhibition and its Effect on Lipid and Drug Transfer

As a fourth modification, human plasma was pre-incubated with the CETP inhibitor torcetrapib (26). For this purpose, $10\ \mu\text{L}$ torcetrapib stock solution ($5\ \text{mM}$ torcetrapib in DMSO) was added to $500\ \mu\text{L}$ human plasma. This mixture was diluted with buffer as described above yielding a final torcetrapib concentration of $50\ \mu\text{M}$ ensuring complete enzyme inhibition. The inhibitor containing plasma was pre-incubated at 37°C for 12 hours. Afterwards liposomes were added and experiments were proceeded as described above. To evaluate any effect of the solvent DMSO, $10\ \mu\text{L}$ of the pure solvent was added to $500\ \mu\text{L}$ human plasma, diluted with buffer and proceeded as described above. Furthermore, the effect on rat plasma and the influence of torcetrapib on lipid and drug transfer in rat plasma was investigated using the same protocol.

Liquid Scintillation Counting (LSC)

Samples were transferred to 5 ml scintillation vials and mixed with a ready-to-use liquid scintillation cocktail (Rotiszint® eco plus, Carl Roth, Karlsruhe, Germany) in a ratio of 1:2 by vortexing the vials for at least 30 sec. As a reference $20\ \mu\text{L}$ of non-separated samples and $5\ \mu\text{L}$ of the pure liposome samples were mixed with $500\ \mu\text{L}$ buffer and 2 ml cocktail. Radioactivity was determined by LSC using a Tricarb 2800 TR LSC (PerkinElmer, Rodgau, Germany). Radioactivity, expressed as disintegrations per minute (dpm), was measured over

5 min. A blank ($500\ \mu\text{L}$ buffer in 2 ml scintillation cocktail) was subtracted from all other samples.

Data Analysis

The obtained elution profiles (radioactivity as percentage of total applied amount for each fraction in dependency of the elution time) were analyzed by Peakfit v4.12 (Systat Software Inc., San Jose, California, USA) according to the residuals method. Area of each peak is a measure of the amount of lipid and drug content in the fractions.

RESULTS

Characterization of Liposomal Formulations

Table I shows the results of the size analysis. Extrusion of liposomes resulted in a mean particle size of approximately 100 nm. Polydispersity indices below 0.1 indicate a narrow uni-modal size distribution for all formulations. Ultracentrifugation of the PEGylated formulation (formulation 3) separated smaller structures into the supernatant, containing $8.8 \pm 1.2\%$ of the added ^{14}C -DPPC label but only traces of ^3H -COE. PEG-PE₂₀₀₀ conjugates are well-known to form few mixed phospholipid/PEG-lipid micelles, which co-exist with liposomes as main structure (27). The different distribution pattern of both lipid labels between the numerically predominant liposomes and the few separated micelles may be caused by their different structure. While ^{14}C -DPPC as a phospholipid based label is distributed like normal DPPC between micelles and liposomes the cholesterol derivative ^3H -COE is solely located in the liposomes due to the curvature reducing effect of cholesterol (28).

Separation of Human Plasma and Liposomes Employing AF4

For method development, evaluation and peak identification, standard solutions of albumin, HDL and LDL were analyzed separately (Fig. 1a). In accordance with these results, the separation of pure human plasma revealed the following elution times: albumin (22 min), HDL (31 min), LDL (52 min) and VLDL (63 min) (Fig. 1b). Peak identity of the lipoproteins HDL, LDL and VLDL was confirmed further by specific staining with Sudan Black B (Fig. 1b). Additionally, on-line DLS measurements of the highly diluted samples revealed particle sizes with z-averages of 8.6 nm for albumin, 12.4 nm for HDL, 25 nm for LDL and 44 nm for VLDL. In Fig. 1c, human plasma and fluid EPC/EPG liposomes containing mTHPC (formulation 2) were analyzed separately. Unfortunately, liposomes showed a peak maximum at 65 min and hence co-elute with a potential VLDL-peak.

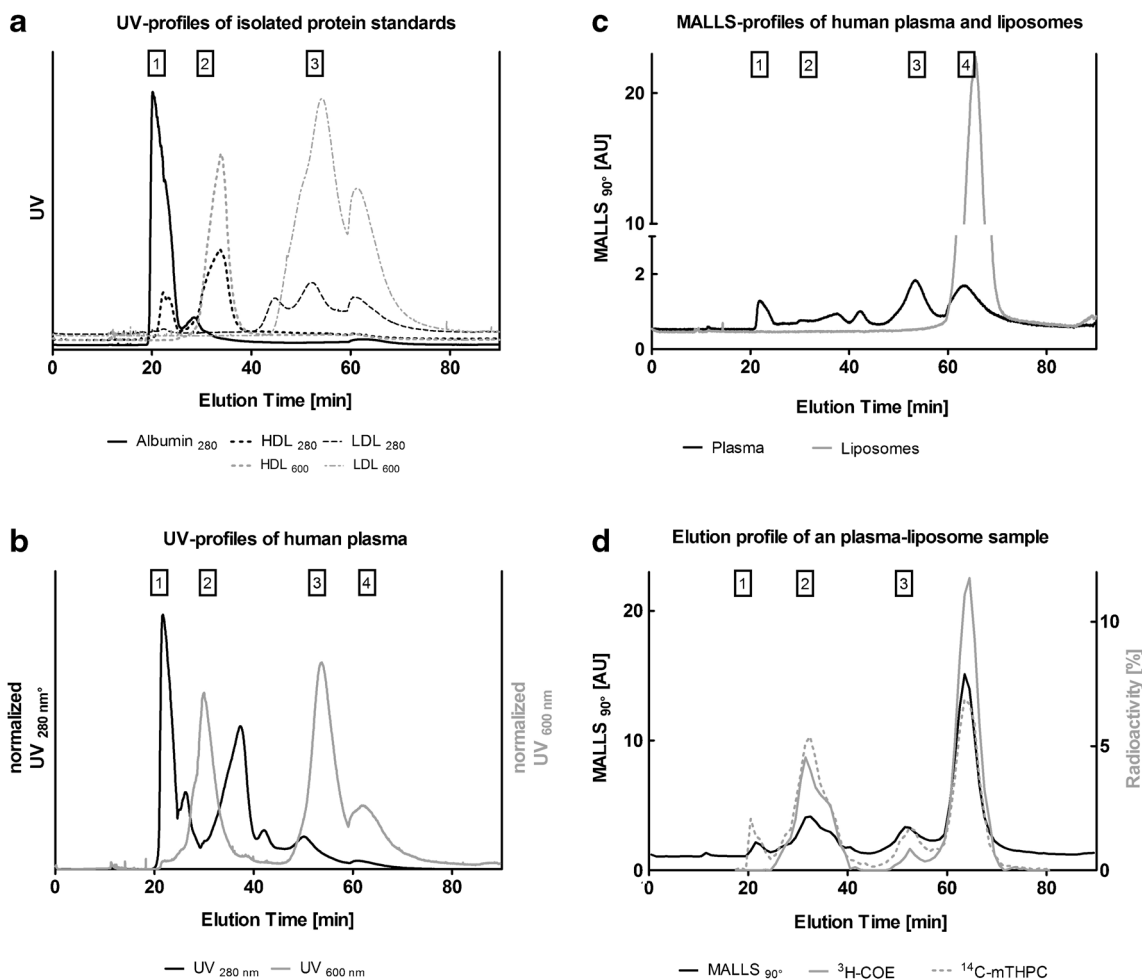


Fig. 1 (a) For peak identification the AF4 elution profiles of the isolated protein standards were recorded (black UV₂₈₀ curves). Based on their size albumin (1), HDL (2) and LDL (3) showed increasing elution times at 20, 31 and 52 min, respectively. With the help of Sudan Black B staining lipoproteins could be specifically detected at 600 nm (grey UV₆₀₀ curves). (b) Separation profile of human plasma (black UV₂₈₀ curves). The elution times obtained with the corresponding standards could be reproduced and the four fractions could be separated from each other. In addition, Sudan Black B staining confirmed peak identity of the lipoproteins (grey UV₆₀₀ curve). (c) MALLS profiles of human plasma and isolated liposomes, which start eluting at 60 min and hence show an overlay with VLDL. (d) Representative separation profile of an incubated plasma-liposome sample. A fluid EPC/EPG formulation (formulation 2 in Table 1) was incubated in human plasma at 37°C for 12 hours. While VLDL and liposomes co-elute after 63 min (4), liposomes could be clearly separated from albumin (1), HDL (2) and LDL (3). The distribution profile of the ³H-COE lipid label and ¹⁴C-mTHPC already indicate that vesicle destruction and drug transfer occur.

Finally, a representative elution profile of an incubated plasma-liposome sample is displayed in Fig. 1d. Formulation 2 was incubated with human plasma at 37°C for 12 hours. Plasma was pre-incubated with torcetrapib for 12 hours prior liposome addition. The distribution curves of lipid and drug label correlate well with the elution profile and 4 well separated fractions can be identified with increasing size as: albumin (1), HDL (2), LDL (3) and liposomes/VLDL (4). These fractions showed a more pronounced signal in the MALLS-detector, which is caused by lipophilic binding of the fluorescent drug.

Recoveries were >90% (mean values) for all lipid labels and 83 ± 13% ($n = 54$) for ¹⁴C-temoporfin ensuring a complete sample elution. Traces of mTHPC could be found in

the cross-flow ($8 \pm 2\%$) and absorbed residues could be seen visually on the membrane. In principal, recorded elution profiles and evaluation of the obtained data indicate a good reproducibility of the experimental results.

Liposome Stability and Lipid Transfer

Effect of Human Plasma Origin on Liposome Stability

Rigid and fluid liposome formulations (formulation 1 and 4) were double labeled with two different lipid labels and incubated with human plasma for 2 hours. The plasma was obtained from five healthy donors (3 male, 2 female). Figure 2 shows that only minor gender-specific differences and

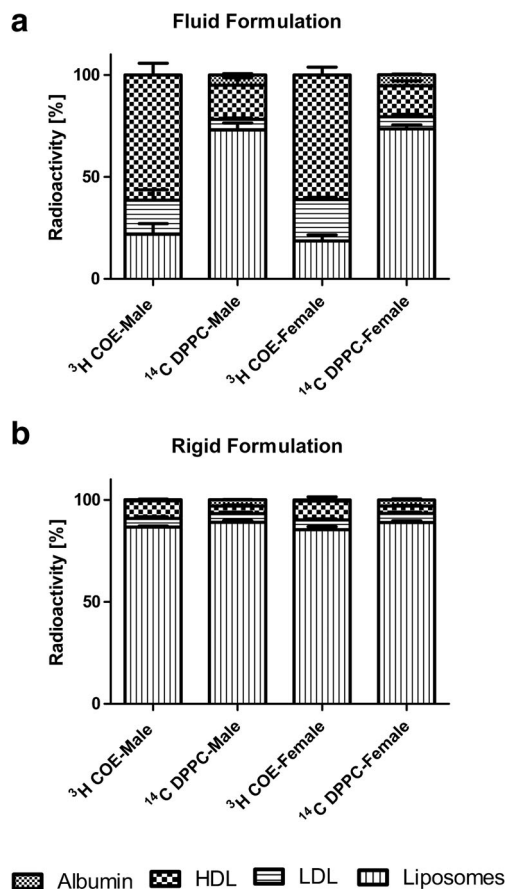


Fig. 2 Distribution profile of ^3H -COE and ^{14}C -DPPC from (a) a fluid formulation, consisting of EPC and EPG (formulation 1) and (b) a rigid formulation, consisting of DPPC and DPPG (formulation 4) to plasma proteins after an incubation for 2 hours at 37°C . Both formulations contained 8 mol% mTHPC. Dependencies of lipid transfer on plasma origin were investigated employing plasma obtained from male and female donors ($n = 6$ for male, $n = 4$ for female plasma).

deviations within each group could be found for both lipid labels. The same was observed for mTHPC transfer out of rigid and fluid liposomes (formulation 2 and 6) (data not shown).

Suitability of Lipid Labels

The liposome formulations in Fig. 2 were simultaneously labeled with ^{14}C -COE, representing a cholesterol based derivative and ^3H -DPPC, a phospholipid based lipid label. Rigid DPPC/DPPG liposomes showed high stability during 2 hours incubation period in human plasma with $86.1 \pm 1.2\%$ ^3H -COE and $88.9 \pm 1.1\%$ ^{14}C -DPPC recovered in the liposome fraction. Even with increasing incubation time, no difference between ^3H -COE and ^{14}C -DPPC with regard to liposome stability could be observed (Fig. 3). In contrast, a significant difference between both labels was found in case of the fluid EPC/EPG formulation. While only a minor part ^3H -COE was left in the liposome fraction ($21.7 \pm 8.3\%$), the ^{14}C -DPPC

label mostly remained in the liposome fraction ($73.3 \pm 3.7\%$). These results may indicate that there is another prominent transfer mechanism for COE in addition to the transfer caused by liposome instability. The addition of 6% mPEG₂₀₀₀-DSPE to an EPC/EPG bilayer increased its stability in human plasma (Fig. 3). It also reduced the observed effects for COE and both employed labels indicate similar stability values. In that case, e.g. 94.93% ^3H -COE and 89.85% ^{14}C -DPPC were recovered in the liposome fraction after 2 hours incubation time (Fig. 3). Slight differences were observed in the transfer pattern of both labels. While ^3H -COE mainly binds to HDL and to a lower extent to LDL, ^{14}C -DPPC also binds to albumin. In addition, COE shows a redistribution from HDL to LDL at longer incubation times (data not shown).

Enzymatic Transfer of COE

In order to clarify the reason for the remarkable difference between ^3H -COE and ^{14}C -DPPC transfer out of EPC/EPG formulations further analysis was focused on a probable enzymatic transfer. Reduction of the incubation temperature for formulation 2 to 4°C could only reduce the lipid transfer for incubation times above 2 hours (Fig. 4). However, the amount of ^3H -COE found in the liposome fraction remained almost constant for all incubation times ($\sim 35\%$) indicating a fast transfer during the separation process e.g. the focus step while the liposomes were in general stable at 4°C in human plasma. mTHPC transfer out of the liposomes was also reduced by a factor of 3. Due to the reduced temperature the Brownian motion is also reduced and hence the diffusion of the drug as well as the collision of liposomes with plasma proteins decreases. Furthermore sample viscosity is increased again leading to a reduction of diffusion and collision processes. Additionally, liposomes (formulation 1) were incubated with isolated albumin and lipoprotein standards. Here, the formulation did not show an enhanced lipid transfer as it was seen after incubation with “complete” human plasma. 100% of the COE lipid label remained in the liposome fraction after 2 hours incubation time of liposomes with pure albumin, 95.06% after incubation with HDL and 88.83% when the formulation was incubated with LDL. In comparison, the values for ^{14}C -DPPC were similar and 95.60, 89.20 and 94.31% were found in the liposome fractions, respectively.

The most remarkable effect on the observed ^3H -COE transfer was caused by the pre-incubation of human plasma with the CETP inhibitor torcetrapib. Especially EPC/EPG liposomes show a distinct increase in the amount of ^3H -COE remaining in the liposome fraction for each time point (e.g. 96 vs 54% for 0.5 hours and 49 vs 18% for 48 hours incubation time). Additionally, the observed redistribution of the lipid label could not be found in case of CETP inhibition.

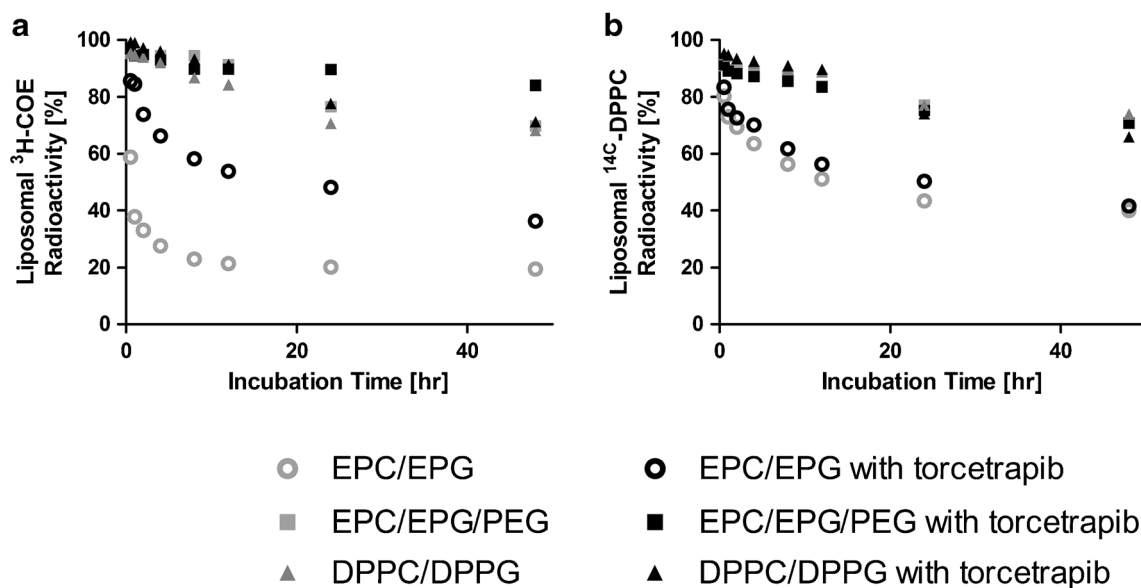


Fig. 3 Proportions of (a) ³H-COE and (b) ¹⁴C-DPPC remaining in in the liposomal bilayer of fluid (formulation 1), PEGylated fluid (formulation 3) and rigid (formulation 4) mTHPC containing liposomes after incubation in human plasma for various time periods at 37°C. The effect of plasma preincubation with the CETP inhibitor torcetrapib on lipid transfer was investigated.

There was also a reduction of ³H-COE transfer (especially after 12 h) in the case of PEGylated EPC/EPG (formulation 3) when CETP was added. Generally, transfer of both lipid labels was lower for PEGylated as compared to non-PEGylated EPC/EPG liposomes indicating that PEGylation increases liposomal stability in human plasma. In pre-treated plasma, 84% COE but only 70% of the DPPC label remained in the vesicles. In contrast, equivalent amounts of both lipid labels were found in the liposome fraction for longer incubation times for non-treated plasma.

Rigid DPPC/DPPG liposomes (formulation 4) were highly stable in human plasma with 90% of the vesicles still being

intact after 12 hours. While the values for ¹⁴C-DPPC were equal in pre- and non-pre-incubated plasma, the amount of ³H-COE in the liposome fraction was slightly higher at each time point when torcetrapib was added.

Figure 3b shows that the enzyme inhibitor had no effect on the ¹⁴C-DPPC transfer and distribution for all formulations. This is also the case for temoporfin (Fig. 4b). Finally we could show, that DMSO as solvent for torcetrapib had no influence on all transfer results (Fig. 4).

Rats are deficient in CETP activity (29). To validate these findings, rat plasma with or without torcetrapib preincubation was mixed with fluid EPC/EPG liposomes

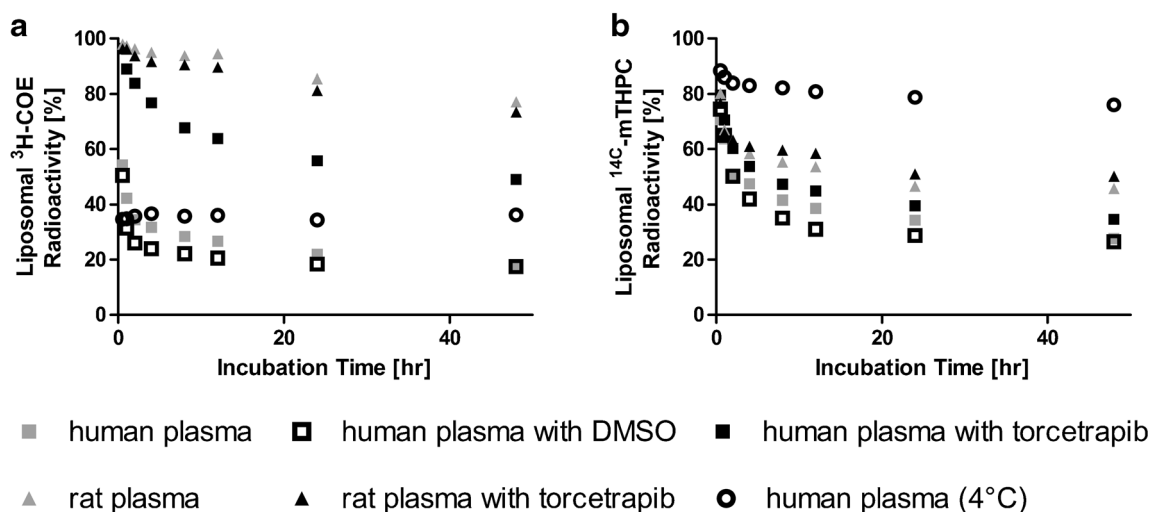


Fig. 4 Proportions of (a) lipid and (b) drug remaining in the liposomes after incubation of mTHPC containing EPC/EPG liposomes (formulation 2) in human or rat plasma for various time periods at 37 and 4°C. The effect of plasma preincubation with the CETP inhibitor torcetrapib and DMSO, temperature and plasma origin on ³H-COE and drug transfer was investigated.

(formulation 2) for 48 hours. Figure 4 shows that the enzyme inhibitor had no effect on the ^3H -COE distribution. Interestingly, the absolute amount of lipid label and ^{14}C -mTHPC recovered in the liposome fraction was significantly higher in rat than in human plasma but the resulting drug-to-lipid ratio was nearly equal (0.65 to 0.7) and kept constant after approximately 4 hours incubation time.

Hydrolysis Effects of DPPC Used as Tracer

DPPC with the label located either on a fatty acid or the choline group have been investigated for their suitability as a potential alternative to ^3H -COE. Both lipid labels were incorporated into a DPPC/DPPG formulation (formulation 5) and the obtained results are displayed in Fig. 5. The results for the

choline labeled ^{14}C -DPPC indicate a liposome stability over 80% after 48 hours not altering during 9 months of storage. In contrast, a constant amount of the fatty acid labeled ^3H -DPPC was initially recovered in the albumin fraction after 0.5 hours incubation time. After 9 months of storage these values were even increased.

Liposome Stability and Drug Transfer in Dependency of the Choice of Lipids

Finally, a fluid EPC/EPG and a rigid DPPC/DPPG formulation each loaded with 8 mol% temoporfin were compared with respect to liposome stability and drug release in human plasma. Plasma was pre-incubated with torcetrapib. The results are shown in Fig. 6. Not surprisingly, the rigid

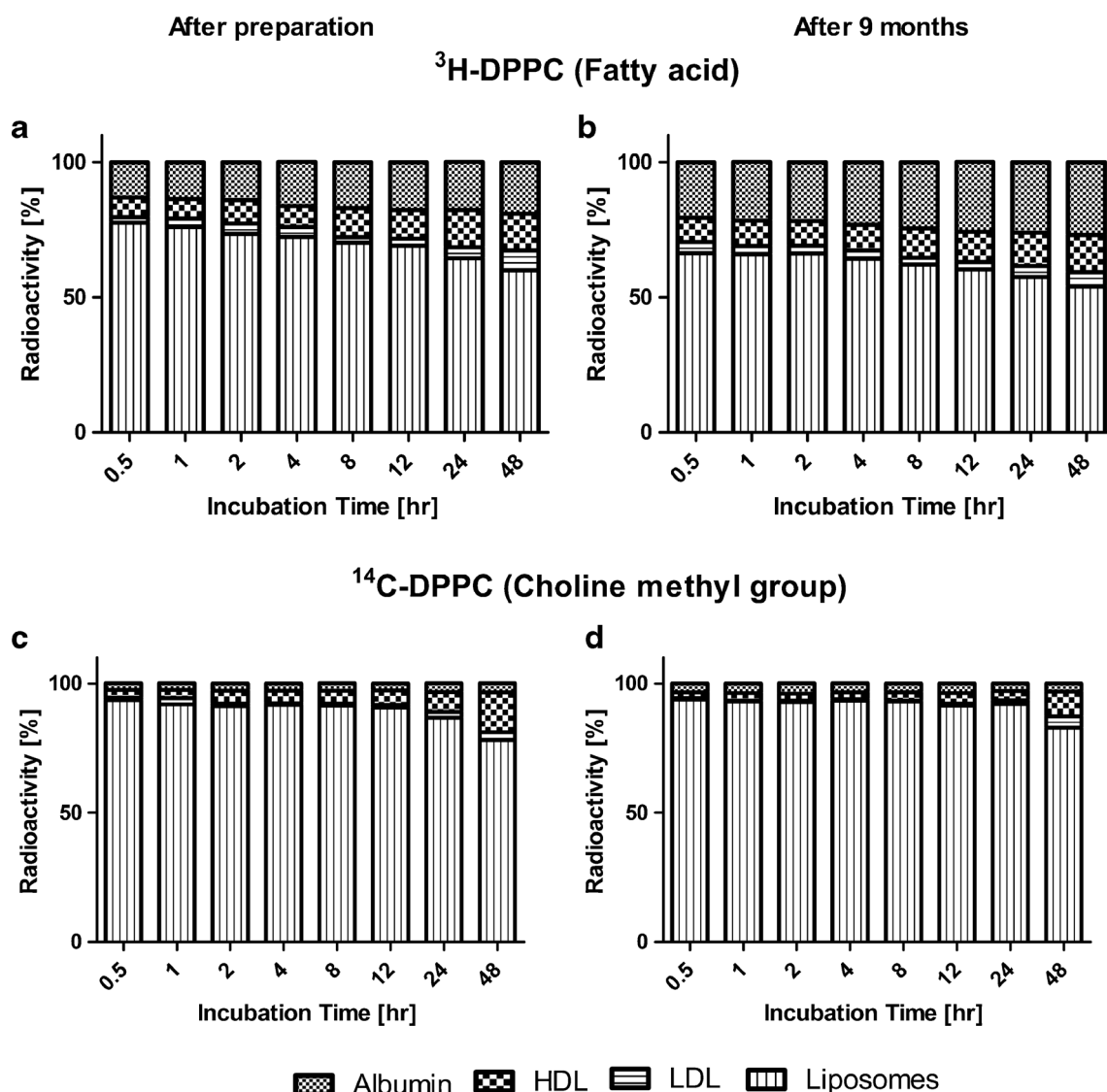


Fig. 5 Distribution profiles of a mTHPC containing DPPC/DPPG formulation, double labelled with two different DPPC labels, whereby the ^3H -DPPC was labelled at a fatty acid and the ^{14}C -DPPC was labelled at a choline methyl group (formulation 5). The effect of phospholipid hydrolysis during preparation and storage over nine months was investigated.

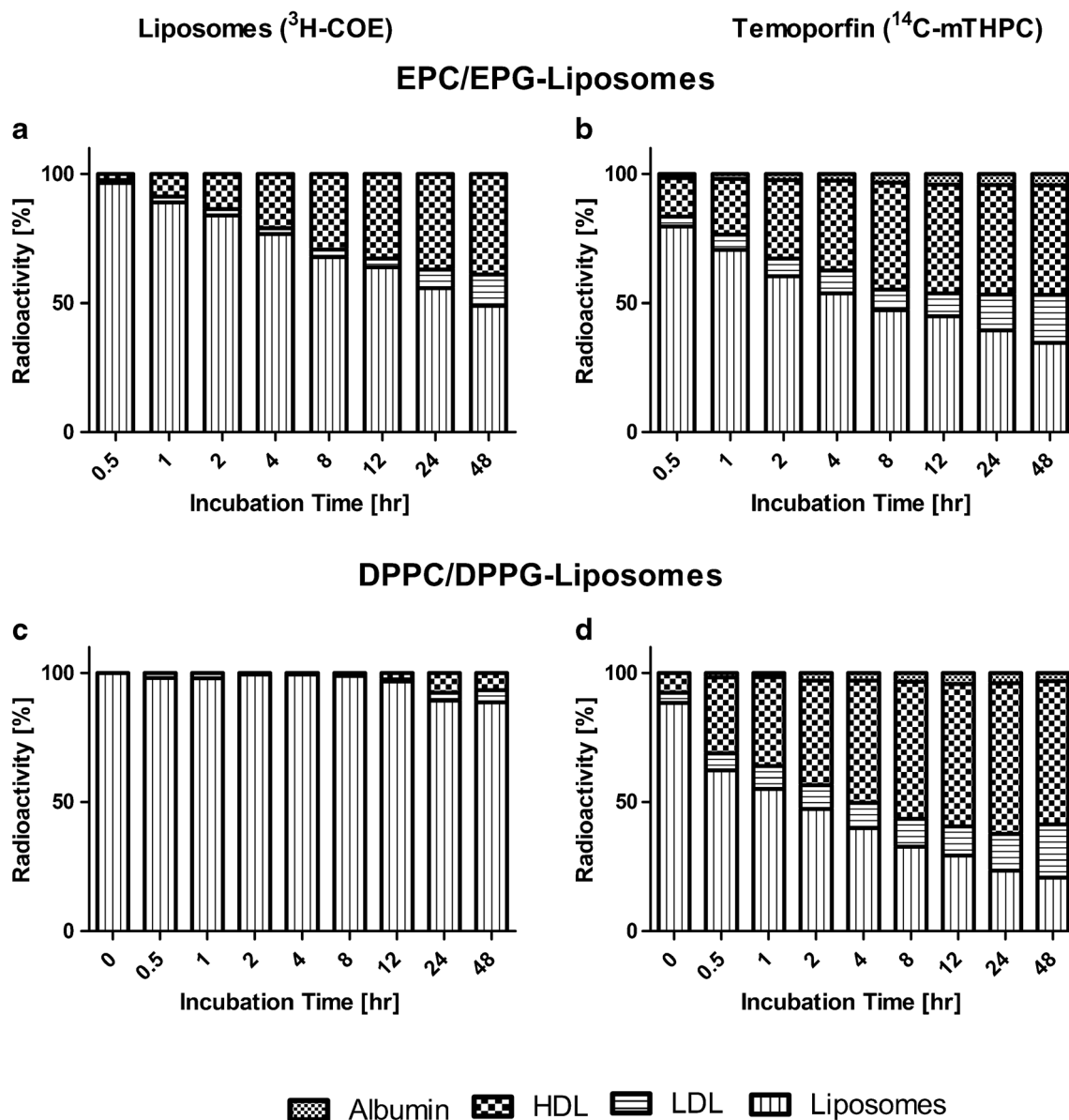


Fig. 6 Distribution profiles of $^3\text{H-COE}$ and $^{14}\text{C-mTHPC}$ from a fluid (formulation 2) and a rigid formulation (formulation 6) after incubation at 37°C for various time periods. Plasma samples were preincubated for 12 hours with torcetrapib.

formulation was always more stable in human plasma than the fluid one. 88% of the vesicles were still intact after 48 hours while only 49% of $^3\text{H-COE}$ were found in the liposome fraction for the fluid formulation. In contrast, the total amount of mTHPC that transferred out of the liposome was higher for DPPC/DPPG liposomes. Here, almost 80% of the drug were transferred to lipophilic domains after 48 hours but only 65% of the applied liposomal drug were released from fluid liposomes, respectively. Size measurements using the MALLS-detector calculated a R_g of the non-incubated fluid liposomes of 37.9 nm. After 0.5 hours, incubation time a R_g of 37.4 nm was calculated for the liposome fraction, which subsequently decreased continuously over the time, ending in R_g values of 35.4 nm after 12 and 32.2 nm after 48 hours.

This shrinking of the liposomes could not be observed for the rigid formulation where the R_g value remained constant at 37.0 ± 0.5 nm over the entire incubation period. Unfortunately, size measurements in highly diluted samples employing MALLS-detection are limited to particles larger than 30 nm. Hence, no reliable size data could be obtained for smaller particles such as proteins and lipoproteins.

For the DPPC/DPPG formulation the effect of system parameters (e.g. conditions during the focus or elution step) were investigated. The results are shown in Fig. 6c and d at incubation time = 0 hours). While the effect on the ^3H transfer was negligible, 11.55% of temoporfin were already transferred to lipoproteins.

DISCUSSION

Suitability of AF4 for Investigations on Plasma-Liposome Interactions

In the past, the separation of human plasma employing a field-flow technique for further analytical purposes was discussed by several groups. Thereby, focus was laid e.g. on plasma protein characterization (30), determination of lipoprotein-cholesterol and triglycerides (16,31) or drug absorption (17). In these cases, plasma protein separation was achieved within 10 to 60 minutes depending on channel geometry and flow-conditions. Separation of liposomes was also achieved within this time range (18–22). However, to our knowledge the separation of liposomes from human plasma by AF4 has not been reported in the literature yet. This approach adds the challenge of an additional separation of liposomes from the lipophilic acceptor domains resulting in a prolonged separation time of 90 minutes.

The resulting elution profiles for human plasma are in good accordance to the profiles shown in literature (15–17). Peaks for albumin, HDL and LDL are well separated. In the light of the experimental conditions (high sample dilution, small particles), particle sizes of these fractions measured by on-line DLS measurements are also in agreement with values published before (32,33). Lipoprotein staining with Sudan Black B identified the HDL and LDL peaks. The peak between was reported in the literature as IgG (30). Liposomes were well separated from the relevant lipophilic acceptor domains. Unfortunately, an overlap in size with VLDL made a separation of both fractions impossible. However, previous studies have shown that VLDL is not considered as a main acceptor for mTHPC (7,11,34,35). In the present study we used plasma obtained from volunteers having fasted for 12 hours ensuring low triglyceride levels and the absence of chylomicrons (36), whose elution would probably also overlap with the liposome fraction. For the same reason, plasma was filtered through a 0.2 µm filter to ensure the absence of larger particles.

In principal, recovery of the sample is strongly dependent on drug lipophilicity (18), which is therefore the limiting factor in this kind of study. Recovery rates of the lipid labels and mTHPC are in good agreement to similar AF4 studies (18,21). While only traces of the lipid label were found in the cross-flow or were absorbed on the membrane, larger amounts of mTHPC could be detected visually on or rinsed through the cellulose membrane. Washing out of the drug and sample absorption has already been reported for albumin, free drug (17) or drug carrier systems (19,37) before. The authors recommended a pre-saturation of the membrane or addition of surfactants (e.g. 0.005% (m/V) Polysorbate 80®) to the carrier liquid to overcome this problem, which occurs mainly for high cross-flow rates as it is the case during the

focus step. In our studies most dye could be recovered in the area around the injection spot. Absorption on the membrane could also result in a transfer of the sample back from the membrane to plasma components during the elution. However, our investigations have shown that this effect plays only a minor role.

During the focus step of 12 minutes sample components were concentrated in a small area and sample interactions could occur. Our studies with a rigid DPPC/DPPG formulation have shown that 11.55% of the drug were released out of the liposomes and transferred to lipoproteins when plasma and liposomes were injected successively. This observed transfer is important to consider for short incubation times but can be neglected if the sample was in contact with plasma for a longer period before.

Suitability of COE as a Lipid Label

A suitable lipid label for these kind of *in vivo* or *in vitro* kinetic studies is incorporated stably in the liposomal bilayer, is not metabolized during the whole experimental period and shows no additional active or passive transfer behavior.

Cholesteryl ethers are metabolically stable (38) and have been used as a lipid tracer for long time in various *in vivo* and *in vitro* studies. Recent studies revealed that this label exchanges very slowly between two lipid membranes (39). However, a very high transfer for the lipid marker out of an EPC/EPG liposome formulation to lipoproteins in human plasma was described previously (7). Investigations in rabbit plasma have shown, that cholesterol esters as well as cholesterol ethers are substrates of the CETP with the esters showing a two times higher transfer rate (40). Rat plasma is deficient in CETP activity. Hence, it is not surprisingly that an enhanced ³H-COE transfer was not observed during *in vivo* experiments in rats (5,41).

Our studies have shown that COE located in fluid liposomal bilayers is indeed a substrate of CETP. Hence, no enhanced COE transfer could be observed when liposomes were incubated with isolated lipoprotein standards or in rat plasma. The pre-incubation of rat plasma with torcetrapib showed also no effect on the lipid label transfer behavior. However, preincubation of human plasma with torcetrapib could inhibit the enzyme induced lipid label transfer. Torcetrapib binds with 1:1 stoichiometry to CETP and 100% of the protein was inhibited at concentrations above 1 µM (26). In our experimental setup, this concentration was exceeded by far and ensured a 100% inhibition of the protein. In human plasma, CETP is mainly bound to HDL and promotes e.g. the redistribution of cholesteryl esters between HDL and LDL (42). Thus, it is explainable that ³H-COE showed a first initial transfer to HDL before it was redistributed to LDL at longer incubation times. This redistribution could not be observed to that extend when CETP was inhibited.

CETP induced cholesterol ether transfer was strongly depending on the bilayer composition. It was clearly reduced for the DPPC/DPPG formulation and delayed for a PEGylated EPC/EPG formulation. Obviously, for these formulations the protein was hindered by the rigidity of the bilayer and the PEG-chains to “suck” the lipid label out of bilayer.

Torcetrapib inhibits phospholipid transfer as well (26). However, ^{14}C -DPPC transfer was not affected by CETP inhibition. mTHPC is not a substrate of CETP and hence, no effect of CETP inhibition was observed.

Further studies are necessary to investigate the effect of the bilayer composition on lipid label transfer more in detail.

DPPC as a Potential Alternative to COE

In the past, radioactive phospholipids have been used as lipid markers for investigations on interactions between liposomes and plasma components (6,43,44). This seems to be a reasonable approach as they are original components of the liposomal bilayer.

However, phospholipids in human plasma are a substrate of the phospholipid transfer protein (PLTP). This protein is capable to transport phospholipids from a liposome bilayer to lipoproteins such as HDL (44). In order to bind a phospholipid the protein has to disturb the membrane locally. Hence, the transfer rate is strongly dependent on bilayer composition or membrane curvature. Consequently, transfer out of a bilayer in liquid crystalline state is easier to achieve in comparison to bilayers in gel state (6,45). Protein mediated phospholipid transfer to lipoproteins can falsify results for liposome integrity (46). In general, active exchange processes are unwanted but as PLTP might also play a role in liposome disintegration, an inhibition would also influence liposome stability. As kinetic profiles for DPPC obtained in our studies were similar to those of COE (in presence of torcetrapib) we conclude that these effects played only a minor role. However, further studies are necessary to get a better overview.

Furthermore, hydrolysis effects also play an important role for the fate of phospholipids in human plasma. In principal, hydrolysis of phospholipids is temperature dependent and increased at higher temperatures (47). Long-time storage or frequent freezing and thawing of phospholipids as well as liposome preparation at higher temperatures can also lead to phospholipid hydrolysis. Additionally, hydrolysis processes also occur in human plasma either intrinsically or due to enzymatic activity (44). For instance, DPPC can be hydrolyzed by lipoprotein lipase (LPL) (48). The resulting lysophospholipids and fatty acids have an influence on the stability, bilayer permeability and hence, release behavior of liposomes (49). Thus, hydrolysis processes of a phospholipid based label also have to be taken into account when being employed as a marker for liposomal stability in human plasma. Depending on the location of the radioactive label the behavior after hydrolysis can

vary strongly. This was confirmed by the DPPC double labeled rigid formulation (formulation 5). DPPC labels with the radioactivity located at the choline group show a different transfer behavior after hydrolysis processes in comparison to phospholipid labels with the radioactivity located at the fatty acid. Free fatty acids show higher affinities to albumin (50). Hence, radioactive labeled free fatty acids can be initially transferred to albumin at short incubation times (Fig. 5). This percentage increases with storage time. In contrast, phospholipids as well as lysophospholipids are transported in human blood bound to lipoproteins and hence, show higher affinities to lipoproteins, mainly HDL in comparison to free proteins (51). Finally, differences in the transfer behavior of both DPPC markers could be seen not only for 0.5 hours of incubation time but also for longer incubation times. Fatty acid labeled DPPC showed a higher transfer rate in comparison to choline labeled DPPC resulting in an apparent lower liposome stability.

Effect of Bilayer Composition on Liposome Stability and Drug Transfer

Among the investigated formulations, non-PEGylated EPC/EPG liposomes showed the lowest stability in human plasma. Bilayers in liquid crystalline state can be disturbed more easily by foreign molecules than bilayers in gel state. Hence, disintegration of vesicles in plasma due to interactions with plasma proteins is enhanced for fluid liposomes (44,45). Here, PLTP induced transport of phospholipids to lipoproteins is only one described mechanism. Consequently, the vesicles may shrink in size. We were able to observe this effect for the fluid EPC/EPG formulation by on-line coupled size measurements. One approach to increase liposomal stability is a change towards gel-phase phospholipids. However, it has been shown in several studies that these bilayers are less capable to hold the lipophilic photosensitizer mTHPC, which is rapidly transferred to potential lipophilic acceptor domains such as lipoproteins or vesicles (5,10). Therefore, PEGylation of the fluid bilayer seems to be a promising attempt to combine both—stability in human plasma and retention of the drug in the liposomes. Due to steric hindrance effects, interactions with plasma proteins can be reduced in order to achieve an increased blood circulation time (9,52). In our case, addition of mPEG₂₀₀₀-DSPE to an EPC/EPG bilayer led to a significant reduction of liposome disintegration. Similar studies demonstrated the same effect for similar formulations. Here, it was also shown that the addition of phosphatidyl oligoglycerols as a potential alternative to PEGylated phospholipids yields in an increased stability *in vivo* and *in vitro* (5,11,35).

Regardless of the liposome formulation, mTHPC preferably binds to HDL and LDL during incubation due to hydrophobic interactions (53). Binding to albumin is less pronounced

and only slightly increasing for longer incubation times. Recent studies with a similar experimental setup but sample separation by SEC showed a similar release profile of mTHPC for a fluid and a rigid formulation (7). Hence, separation of a plasma-liposome sample by AF4 seems to be a potential alternative to established methods.

CONCLUSIONS

Our study introduces a novel approach to measure liposome-plasma protein interactions based on size by employing AF4. Thereby, we obtained a good separation profile for liposomes and the three potential main acceptor domains albumin, HDL and LDL within a relative short time of 90 minutes. On-line coupling of various detectors enabled a simultaneous analysis of the sample e.g. for size determinations. Furthermore, the effect of the bilayer composition on liposome stability and drug release was investigated. As reported before, rigid liposomes and PEGylated fluid liposomes showed higher apparent stabilities in human plasma than a non-PEGylated fluid formulation. In contrast, the fluid formulation was more capable to hold mTHPC within the bilayer. Hence, bilayer composition has a high influence on stability and drug release of a liposomal formulation in human plasma and membrane modifications should be considered to achieve an appropriate behavior. This should be investigated deeper in future experiments

The “Right” Lipid Label

Requirements for a suitable lipid label were stated as stable incorporation in the liposomal bilayer, no metabolization and no additional transfer behavior except for liposomal instability. The combination of COE with torcetrapib offers a promising approach to fulfill these criteria. Nevertheless, studies on possible interactions with the inhibitor are necessary for each liposomal incorporated drug. DPPC labels are potential alternatives to this setup. However, active transport and hydrolysis (also during storage of the label) are two major limiting factors which have to be considered and analyzed prior to each experiment. Among the used DPPC labels, choline labeled DPPC has to be favored, even though it suffers the drawback of high costs.

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