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In Vivo Quantitative Understanding of PEGylated Liposome's Influence on Brain Delivery of Diphenhydramine

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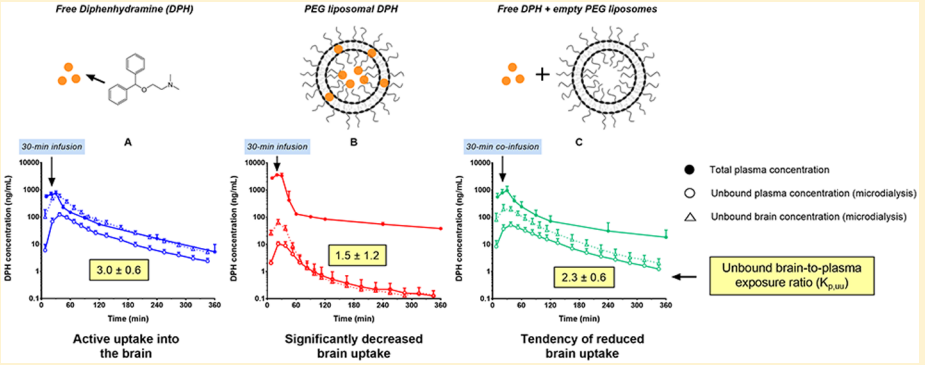
Supporting Information



ABSTRACT: Despite the promising features of liposomes as brain drug delivery vehicles, it remains uncertain how they influence the brain uptake in vivo. In order to gain a better fundamental understanding of the interaction between liposomes and the blood−brain barrier (BBB), it is indispensable to test if liposomes affect drugs with different BBB transport properties (active influx or efflux) differently. The aim of this study was to quantitatively evaluate how PEGylated (PEG) liposomes influence brain delivery of diphenhydramine (DPH), a drug with active influx at the BBB, in rats. The brain uptake of DPH after 30 min intravenous infusion of free DPH, PEG liposomal DPH, or free DPH + empty PEG liposomes was compared by determining the unbound DPH concentrations in brain interstitial fluid and plasma with microdialysis. Regular blood samples were taken to measure total DPH concentrations in plasma. Free DPH was actively taken up into the brain time-dependently, with higher uptake at early time points followed by an unbound brain-to-plasma exposure ratio ($K_{p,uu}$) of 3.0. The encapsulation in PEG liposomes significantly decreased brain uptake of DPH, with a reduction of $K_{p,uu}$ to 1.5 ($p < 0.05$). When empty PEG liposomes were coadministered with free drug, DPH brain uptake had a tendency to decrease ($K_{p,uu}$ 2.3), and DPH was found to bind to the liposomes. This study showed that PEG liposomes decreased the brain delivery of DPH in a complex manner, contributing to the understanding of the intricate interactions between drug, liposomes, and the BBB.

KEYWORDS: *nanocarrier, liposome, blood-brain barrier, brain uptake, microdialysis, diphenhydramine*

INTRODUCTION

The presence of the blood−brain barrier (BBB) poses a huge challenge for efficient drug delivery to the central nervous system (CNS). The use of nanocarriers (e.g., liposomes, nanoparticles) as drug delivery vehicles may provide opportunities to enhance the uptake of drugs across the BBB.[1−3] However, it remains unclear how nanocarriers influence the BBB transport of payload in vivo. Moreover, given that the transport of drugs at the BBB can be dominated by either active influx or active efflux, it is uncertain whether brain uptake of drugs with different BBB transport properties can be affected differently when they are nanoencapsulated.

To answer these questions, a pharmacokinetic (PK) evaluation separating the released drug from the drug remaining in the nanocarrier is of great importance. Microdialysis plays an important role in this regard, providing unique

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and quantitative information, as it allows the different in vivo processes to be separated by measuring the released, unbound drug concentrations in both brain and blood over time together with regular blood sampling for total drug concentrations in plasma.[4] Thus, the impact of nanocarrier on the BBB transport of drugs can be specifically studied without being confounded by potential blood contamination in brain tissue. In two recent studies using microdialysis, we showed that encapsulation in PEGylated (PEG) liposomes could improve the brain uptake of both small molecule (methotrexate) and peptide (DAMGO) payloads to different extents.[5,6] Methotrexate (MTX) and DAMGO demonstrate active efflux at the BBB when administered as free drug, as described by the unbound brain-to-plasma concentration ratio ($K_{p,uu}$) of 0.1 and 0.05, respectively. After liposomal encapsulation, their $K_{p,uu}$ values were increased 3- and 2-fold, respectively. Additional modeling analysis indicates that the most likely reason for enhanced brain delivery of DAMGO is a fusion of liposomes with the BBB cell membrane.[7] This fusion process may to some extent bypass the efflux transporters responsible for the limited CNS drug access. However, in order to thoroughly understand how liposomes interact with the BBB, it is necessary to test other payload drugs with different BBB transport properties, e.g., drugs with active influx at the BBB, indicated by $K_{p,uu} > 1$.

If the nanocarrier could potentially lead to transporter circumvention when they interact with the BBB, they would probably not only neutralize the role of efflux transporters but also influence influx transport, possibly decreasing brain delivery of drugs with active uptake at the BBB. A recently published microdialysis study has indicated that this hypothesis may be valid by showing a reduced brain uptake of quetiapine when the drug was loaded into lipid-core nanocapsules, with a reduction in $K_{p,uu}$ from 1.55 to 0.94.[8] Nevertheless, there is currently no quantitative evidence suggesting that liposomes behave similarly to nanocapsules.

To assess how influx drug transport at the BBB could be influenced by liposomes, diphenhydramine (DPH) was selected as a model compound. DPH was reported to have a $K_{p,uu}$ of 5.5, indicating that active influx dominates its transport at the BBB.[9] The active uptake of DPH into the brain is mediated by the proton-coupled organic cation antiporter system.[10] DPH is also a compound that is suitable to study with microdialysis because of the minimal sticking to tubing and probes.[9−12] In the present study, PEGylated liposomes containing egg yolk phosphatidylcholine (EYPC) were selected, since this formulation with high fluidity was suggested to interact with the BBB more easily through the proposed fusion mechanism, compared to a more rigid formulation.[5]

The aim of this study was to investigate the influence of PEGylated liposomes on the uptake of DPH across the BBB in vivo. For this purpose, quantitative microdialysis in blood and striatum was performed together with regular blood sampling. This enables simultaneous monitoring of the concentration−time profiles of released, unbound DPH in both brain and plasma, as well as total DPH in plasma. This study, combined with the previous work on MTX and DAMGO,[5,6] can provide quantitative elucidation on how the liposomes potentially affect the brain delivery of drugs with active influx or active efflux, which helps the in-depth understanding of how liposomes interact with the BBB.

MATERIALS AND METHODS

Materials. DPH hydrochloride (HCl) powder and deuterated DPH (DPH-D3) solution (100 μg/mL) were purchased from Sigma-Aldrich (Steinheim, Germany). EYPC and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-conjugated polyethylene glycol MW 2000 ($mPEG_{2000}$-DSPE) were obtained from Lipoid (Cham, Switzerland). Cholesterol was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The Ringer solution, consisting of 145 mM NaCl, 0.6 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 0.1 mM ascorbic acid in 2.0 mM phosphate buffer (pH 7.4), was prepared in-house. Acetonitrile, ammonia, formic acid, and glacial acetic acid were of analytical grade (Merck, Darmstadt, Germany). The water was purified with a Milli-Q Academic system (Millipore, Bedford, MA, USA).

Liposome Preparation and Characterization. PEG liposomal DPH and empty PEG liposomes were prepared using an ethanol injection method with a pre-insertion of $mPEG_{2000}$-DSPE. In short, 100 mM EYPC, 66 mM cholesterol, and 8.7 mM $mPEG_{2000}$-DSPE (5 mol %) were first dissolved in absolute ethanol. Then, 9.6 mL of lipid mixture was mixed with 30.4 mL of a solution of 25 mg/mL DPH HCl in Milli-Q water for the drug-loading liposomes or saline for the empty liposomes at 60 °C. The produced liposomes were extruded stepwise through 200/200 and 200/100 nm Whatman filters (Instruchemie, Delfzijl, The Netherlands) to reduce and unify particle size. Nonencapsulated DPH was removed by ultrafiltration on a Cogent μScale Tangential Flow Filtration System (Merck Millipore) using a Pellicon XL 50 Cassette equilibrated with saline. The purified liposomes were sterile filtered using 0.2 μm filters, and aliquots were stored at 4 °C until further use.

After releasing the DPH from the liposomes with acetonitrile, the encapsulated drug was quantified using an HPLC-UV method. Briefly, chromatographic separation was carried out on a Xbridge C18 column (150 mm × 4.6 mm, 3.5 μm) (Waters, CA, USA) using a Shimadzu 20A ultrafast liquid chromatography system (Shimadzu, Kyoto, Japan) under isocratic elution with a mobile phase consisting of acetonitrile:sodium dihydrogen phosphate (pH 3.5 adjusted with glacial acetic acid) (40:60, v/v). The flow rate was 1 mL/min, and the column temperature was set at 23 °C. The UV wavelength for detection was set at 205 nm. The EYPC concentrations in the liposome samples were analyzed using HPLC with an evaporative light-scattering detector (ELSD) (Alltech, Ridderkerk, The Netherlands). A Kinetex C18 column (150 mm × 4.6 mm, 2.6 μm, Phenomenex) was used for analysis with the column temperature set to 45 °C. The chromatographic separation was performed using a gradient elution of 0−10% mobile phase A (0.1 M ammonium acetate, pH 6.0) and mobile phase B (methanol) at a flow rate of 1.5 mL/min, with the total run time being 20 min. The nitrogen gas flow of the ELSD was set at 1.5 mL/min with a temperature of 80 °C. A Malvern Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK) was used to measure the size of the liposome.

In Vitro Drug Release. The in vitro stability of PEG liposomal DPH in both phosphate-buffered saline (PBS) and rat plasma was tested. For the experiment, 10 μL of liposome solution was added into 40 μL of either PBS or rat plasma, and the mixture was incubated at 37 °C for 48 h. Samples were taken at 0, 1, 2, 4, 8, 24, and 48 h during the incubation. Each

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sample was first diluted with 100 μL of PBS before loading 100 μL of the diluted sample onto a Zebaspin desalting column (Thermo Scientific, Rockford, IL, USA) pre-equilibrated with PBS. After that, the encapsulated DPH in the purified sample was released by adding 100 μL of acetonitrile. The concentration of DPH was measured using the above-mentioned HPLC-UV assay. The amount of liposome-encapsulated DPH at each time point was presented as the percentage of the initial value at time 0 (T0).

Animals and Surgery. The animal procedures and study protocols were approved by the Uppsala Regional Animal Ethics Committee, Uppsala, Sweden (C13/14). Male Sprague−Dawley rats were purchased from Taconic (Lille Skensved, Denmark). The rats were allowed to acclimatize for 7 days before the experiments in temperature- and humidity-controlled conditions with a 12 h light/dark cycle and unrestricted access to food and water. The rats weighed 245−285 g on the experimental day.

The rats were anesthetized through inhalation of 2.5% isoflurane (Isoflurane Baxter, Baxter Medical AB, Kista, Sweden), combined with 1.5 L/min oxygen and 1.5 L/min nitrous oxide. The body temperature of the rats was kept at 38 °C by a CMA/150 temperature controlled heating pad (CMA, Stockholm, Sweden). PE-50 cannulas (MicLev, Malmö, Sweden) were inserted into the left and right femoral veins for drug administration and the left femoral artery for blood sampling. The cannulas were pretreated with 100 IU/mL heparin in saline to avoid clotting. Then, a flexible CMA/20 microdialysis probe with a 20 kDa cutoff and 10 mm polyarylethersulfone (PAES) membrane (CMA, Stockholm, Sweden) was inserted into the right jugular vein to measure the released, unbound DPH concentrations in blood and fixed to the pectoral muscle by two sutures. For sampling in brain interstitial fluid (ISF), a CMA/12 guide cannula was first implanted into striatum through a stereotaxic instrument (David Kopf Instruments, Tujunga, USA), with a position of 2.7 mm lateral and 0.8 mm anterior to the bregma and 3.8 mm ventral to the brain surface. After having been fixed to the skull with a screw and dental cement (Dentalon Plus Heraeus, Germany), the guide cannula was cautiously substituted by a CMA/12 microdialysis probe with a 20 kDa cutoff and 3 mm PAES membrane (CMA, Stockholm, Sweden). All catheters were passed subcutaneously to the posterior surface of the neck. After surgery, the rats were individually placed in a CMA/120 system for freely moving animals and were given 24 h for recovery before the experiments started.

Experimental Procedures. On the day of experiment, microdialysis probes were constantly perfused with Ringer solution containing 10 ng/mL DPH-D3 as the recovery calibrator at a flow rate of 1 μL/min, using a CMA/100 precision infusion pump (CMA, Stockholm, Sweden). The perfusion started 90 min before the start of the i.v. administration. Fluorinated ethylene propylene tubing (CMA, Stockholm, Sweden) were used as inlet and outlet tubing. The in vivo recovery of the probe was individually measured throughout the whole study period in accordance with retrodialysis.[13] Before and after sample collection, all sampling vials were weighed to examine the flow through the probe during the experiment.

To compare the PK profiles between PEG liposomal DPH and free DPH, a 30 min short infusion regimen was used. For the liposomal group (n = 8), the rats were intravenously administered 4.5 mg/kg (150 μg/min/kg) of PEG liposomal DPH through the left femoral vein, using a Harvard 22 pump (Harvard Apparatus Inc., Holliston, MA). For the "free drug group" (n = 7), rats received an intravenous administration of free DPH in saline at the same dose as the PEG liposomal DPH, as a reference administration. One rat in the free DPH group was excluded due to a microdialysis sampling issue from the brain probe. To more thoroughly understand the liposomal influence on the PK profiles of DPH, a coadministration group (n = 4) was additionally included, in which free DPH (4.5 mg/kg) and empty PEG liposomes (same EYPC dose as PEG liposomal DPH) were injected simultaneously into the left and right venous catheters as a 30 min infusion, respectively. The microdialysate fractions from brain ISF and blood were collected in polypropylene vials (AgnThos, Lidingö, Sweden) every 15 min (∼15 μL) after the start of the retrodialysis period until 120 min after the start of the infusion, and then every 30 min (∼30 μL) for 4 h. The midpoint time in each collection interval was used to plot the concentration−time curve. Blood samples (∼200 μL) were withdrawn into preheparinized polypropylene tubes before the infusion started and at 10, 20, 30, 45, 60, 90, 120, 240, and 360 min after the start of the infusion. After immediate centrifugation at 10000 rpm (7200g) for 5 min, the plasma was transferred to clean polypropylene tubes. All samples were stored at −20 °C until analysis.

Sample Analysis. An ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method was used for quantifying DPH and DPH-D3 in microdialysate and plasma. For sample preparation, 50 μL of plasma was precipitated with 150 μL of acetonitrile containing 100 ng/mL DPH-D3 as the internal standard (IS). After being vortexed and centrifugated for 3 min at 13 000 rpm, a volume of 10 μL of the supernatant was further diluted with 500 μL of the mixture of mobile phase A and B (90/10; v/v). Five microliters was then injected onto the UPLC-MS/MS system. An aliquot of a 10 μL microdialysis sample was transferred to the injection vial containing 90 μL of the same mobile phase mixture, before injecting 5 μL of the diluted microdialysate onto the UPLC-MS/MS system.

Chromatographic separation was carried out on an ACQUITY BEH C18 column (50 mm × 2.1 mm, 1.7 μm) protected by an ACQUITY BEH C18 guard column (10 mm × 2.1 mm, 1.7 μm) (Waters, CA, USA) using a Waters ACQUITY UPLC system (Waters, CA, USA). Mobile phase A consisted of 90% 5 mM ammonium formate buffer (pH 3.4) and 10% acetonitrile, and mobile phase B consisted of 90% acetonitrile and 10% 5 mM ammonium formate buffer (pH 3.4). The gradient elution started at 10% B for 0.5 min, then increased linearly to 40% B in 0.5 min, and maintained at 40% B for 1.5 min, before returning to the initial condition for equilibration. The flow rate was 0.3 mL/min, and the total run time was 3 min. A Quattro Ultima triple quadrupole mass spectrometer was used for the MS/MS detection (Waters, Milford, MA, USA), and MassLynx software version 4.1 (Waters, Milford, MA, USA) was utilized for data acquisition and processing. The detection of DPH and DPH-D3 was performed in a positive electrospray mode using multiple reaction monitoring transitions of 256.2 → 167.0 for DPH and 259.2 → 167.0 for DPH-D3. The standard curve with a range of 1−5000 ng/mL DPH was used for plasma samples. For the microdialysis samples, the standard curve for DPH-D3 ranged from 0.1 to 20 ng/mL. The standard curves of DPH were 1−500 ng/mL for the samples from the free drug group and 0.1−

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50 ng/mL for the samples from the liposomal group. Using $1/x^2$ weighting, the coefficient of determination (R^2) was higher than 0.99 for all the standard curves. The quality controls were accurate and precise in all runs with a coefficient of variation being lower than 15% .

Data Analysis. No adsorption of DPH and DPH-D3 to microdialysis probes and tubing was observed. The in vivo recovery for each microdialysis probe was calculated as

$$\text{Recovery} = \frac{(C_{\text{calibrator,in}} - C_{\text{calibrator,out}})}{C_{\text{calibrator,in}}} \quad (1)$$

where $C_{\text{calibrator,in}}$ is the concentration of DPH-D3 in the microdialysis perfusate determined from triplicates before and after the experiment, and $C_{\text{calibrator,out}}$ is the average concentration of DPH-D3 in the collected dialysate during the whole experiment.

The probe recoveries were stable throughout the experiments. The average recovery was 0.37 ± 0.05 and 0.85 ± 0.06 for the brain and the blood probes, respectively.

The unbound concentration (C_u) of DPH in blood and brain ISF was calculated using the following equation

$$C_u = \frac{C_{\text{dialysate}}}{\text{Recovery}} \quad (2)$$

where $C_{\text{dialysate}}$ is the concentration of DPH in the collected microdialysis samples, and recovery is the individual average measurement from either the brain or blood probe.

The PK parameters of total plasma concentration of DPH were estimated on the basis of noncompartmental analysis. The clearance (CL) and volume of distribution at steady state (V_{ss}) for each individual rat were estimated according to the following equations

$$\text{CL} = \frac{\text{Dose}}{\text{AUC}_{0\to\infty}} \quad (3)$$

$$V_{ss} = \frac{R_0 T \times \text{AUMC}_{0\to\infty}}{\text{AUC}_{0\to\infty}^{\ 2}} - \frac{R_0 T^2}{2 \times \text{AUC}_{0\to\infty}} \quad (4)$$

where R_0 is the rate of infusion of free or PEG liposomal DPH, and T is the duration of the infusion. The area under total plasma concentration versus time curve ($\text{AUC}_{0\to\infty}$) and the area under the first moment versus time curve ($\text{AUMC}_{0\to\infty}$) were calculated using the linear trapezoid method. The residual areas for $\text{AUC}_{0\to\infty}$ and $\text{AUMC}_{0\to\infty}$ were calculated as $C_{\text{last}}/\lambda_z$ and $C_{\text{last}}t_{\text{last}}/\lambda_z + C_{\text{last}}/\lambda_z^2$, respectively, where C_{last} is the concentration measured at the last sampling point, and λ_z is the terminal rate constant estimated from the slope of the 3 last concentrations observed. The terminal half-life ($t_{1/2}$) was calculated as $\ln(2)/\lambda_z$.

The unbound fraction of DPH in plasma (f_u) after the infusion of free DPH was calculated as $f_u = \text{AUC}_{\text{u,plasma}}/\text{AUC}_{\text{tot,plasma}}$, where $\text{AUC}_{\text{u,plasma}}$ and $\text{AUC}_{\text{tot,plasma}}$ represent the area under the unbound drug plasma concentration, calculated from microdialysis sampling, and the area under the total plasma concentration versus time curves, respectively. The ratio of unbound to total DPH in plasma after the infusion of PEG liposomal DPH was also calculated.

The brain uptake of DPH after administering free or liposomal DPH was described by the unbound brain-to-plasma exposure ratio, $K_{\text{p,uu}}$,[14,15] calculated as

$$K_{\text{p,uu}} = \frac{\text{AUC}_{\text{u,brain}}}{\text{AUC}_{\text{u,plasma}}} \quad (5)$$

where the area under the unbound brain concentration ($\text{AUC}_{\text{u,brain}}$) and the area under the unbound plasma concentration ($\text{AUC}_{\text{u,plasma}}$) versus time curves were calculated in two periods (0−60 min and 60−360 min) separately, using the trapezoid method.

All data are presented as mean values ± standard deviation (SD). GraphPad Prism version 7.03 for Windows (GraphPad Software, San Diego CA, USA) was used for the statistical analysis. The PK parameters of total DPH in plasma (CL, V_{ss}, and terminal $t_{1/2}$) among the three groups were compared using one-way ANOVA followed by Turkey's multiple comparison test. The $K_{\text{p,uu}}$ values of DPH in the three groups during the 0−60 and 60−360 min periods were compared using a two-way ANOVA followed by Turkey's multiple comparisons test. A $p < 0.05$ was considered to be statistically significant for all tests.

RESULTS

The average size of PEG liposomal DPH and empty PEG liposomes were 83.6 ± 0.5 and 90.0 ± 0.5 nm, with a polydispersity index of 0.11 ± 0.01 and 0.05 ± 0.01, respectively. Both formulations were comparable in EYPC contents. The encapsulation yielded a DPH concentration of 0.71 mg/mL in the liposomes. After incubation in either PBS or rat plasma, an instability of PEG liposomal DPH was observed (Figure 1). When incubated in PBS, the liposomes

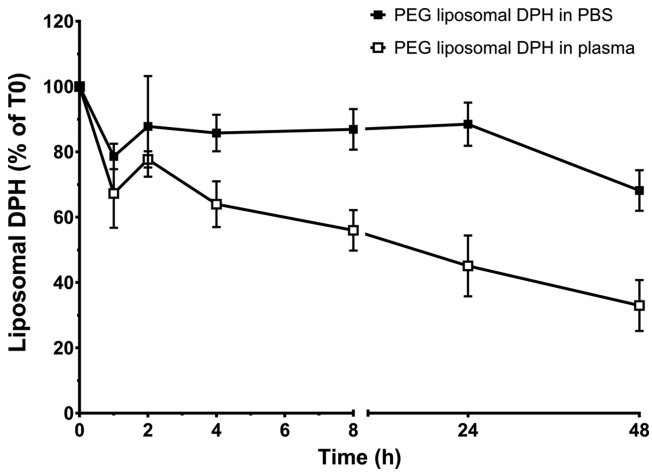

Figure 1. In vitro release of PEG liposomal DPH in PBS and rat plasma at 37 °C (n = 3).

were relatively stable for 24 h. At 48 h, 68% of DPH remained encapsulated relative to the value at T0. A faster drug release was observed when the liposomes were incubated in plasma, with 33% remaining in the liposomes at 48 h.

The concentration−time profiles for DPH after the 30 min i.v. infusion of free DPH, PEG liposomal DPH, or free DPH + empty PEG liposomes are shown in Figure 2. In all groups, biphasic PK profiles were observed for total DPH in plasma. Compared to the free DPH group (Figure 2A), the PEG liposomal DPH resulted in a faster initial decline and a slower elimination phase in plasma (Figure 2B). Thus, the liposomal encapsulation significantly prolonged the terminal $t_{1/2}$. Total plasma CL as well as V_{ss} were significantly decreased relative to

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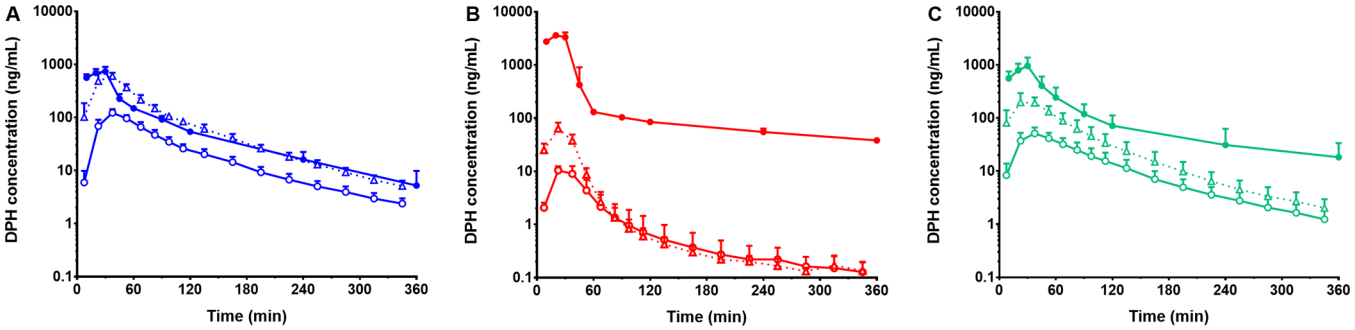

Figure 2. Concentration−time profiles of total DPH in plasma (filled circles), unbound DPH in plasma (open circles), and brain (open triangles) after 30 min i.v. infusion of 4.5 mg/kg of (A) free DPH (n = 7), (B) PEG liposomal DPH (n = 8), or (C) free DPH + empty PEG liposomes (n = 4). Data are expressed as mean ± SD.

Table 1. Estimated PK Parameters of DPH in Rats after the Administration of Free DPH, PEG Liposomal DPH, or Free DPH + Empty PEG Liposomes[a]

	administration groups		
parameters	free DPH	PEG liposomal DPH	free DPH + empty liposomes
terminal $t_{1/2}$ (min)	59.7 ± 5.3	207 ± 9.0**	123.4 ± 39.5**
CL (mL/min/kg)	123 ± 16	31.6 ± 3.7**	90.2 ± 32.3*
V_{ss} (L/kg)	5.2 ± 1.0	2.6 ± 0.5**	7.1 ± 4.0
f_u or $AUC_{u,plasma}/AUC_{tot,plasma}$	0.25 ± 0.05	0.0039 ± 0.0014	0.09 ± 0.02**
$K_{p,uu}$ (0−60 min)	5.4 ± 0.7	5.4 ± 1.0	4.9 ± 2.0
$K_{p,uu}$ (60−360 min)	3.0 ± 0.6	1.5 ± 1.2*	2.3 ± 0.6

[a]The calculation of terminal $t_{1/2}$, CL, and V_{ss} are based on total plasma concentrations. Data are presented as mean ± SD. *p < 0.05 compared to the free DPH group. **p < 0.01 compared to the free DPH group.

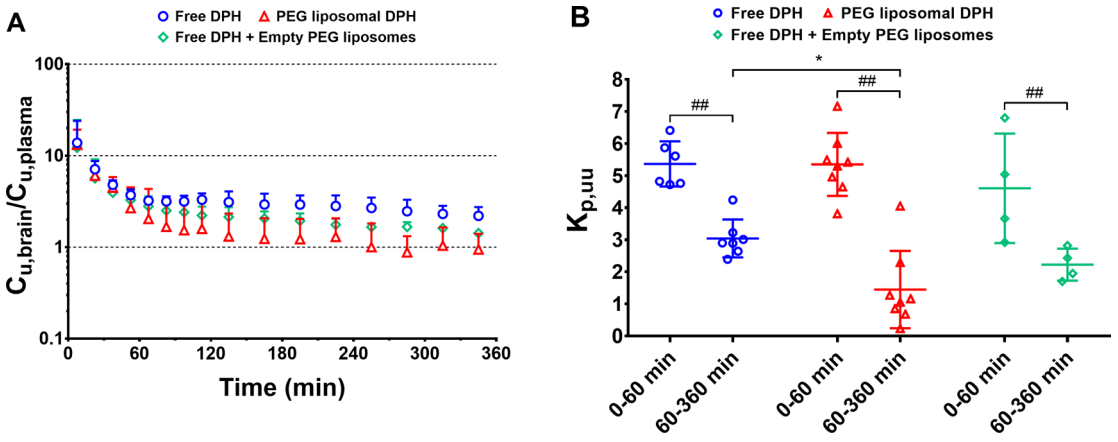

Figure 3. (A) Unbound brain-to-plasma concentration ratio over time after 30 min i.v. infusion of free DPH, PEG liposomal DPH, or free DPH + empty PEG liposomes. Data are expressed as mean ± SD, n = 4−8. (B) Unbound brain-to-plasma exposure ratio ($K_{p,uu}$) divided into the periods 0−60 and 60−360 min. *p < 0.05 between free DPH and PEG liposomal DPH during the 60−360 min period; ##p < 0.01 indicates significant difference between the two periods. n = 4−8.

administering free DPH (Table 1). When free DPH was coadministered with empty PEG liposomes, a slower elimination process was also observed (Figure 2C) compared to administering free drug alone. The coadministration of empty liposomes significantly reduced the total plasma CL and extended the terminal $t_{1/2}$ of DPH (Table 1).

The unbound fraction of DPH in plasma after administration of free drug was 0.25 ± 0.05. This value was significantly decreased in the coadministration group (Table 1), indicating that DPH binds to the liposomes. After administration of PEG liposomal DPH, the total plasma exposure was on average 286 ± 100 times higher than that of the unbound DPH.

The unbound drug concentration in brain was higher than that in plasma throughout the whole sampling period after administration of free DPH, clearly confirming active uptake of DPH at the BBB. However, the $C_{u,brain}/C_{u,plasma}$ changed with time from initially very high values (>10) to stabilizing at around 3 (Figures 2A and 3A). This was also the case after PEG liposomal DPH administration, although with the $C_{u,brain}/C_{u,plasma}$ decreasing even more after the first hour, as clearly seen in Figures 2B and 3A. A similar pattern was also found when empty liposomes were given together with free DPH (Figures 2C and 3A), with the $C_{u,brain}/C_{u,plasma}$ being between that from the other two groups. This was the reason for calculating the $K_{p,uu}$ values in two separate periods (0−60 and

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60−360 min) (Figure 3B). In all three groups, the $K_{p,uu}$ values during 60−360 min were significantly lower than those during the first 60 min ($p < 0.01$, Table 1 and Figure 3B). Comparing the $K_{p,uu}$ values among different groups showed that it was 3.0 ± 0.6 after free DPH administration vs 1.5 ± 1.2 after PEG liposomal DPH ($p < 0.05$, Table 1 and Figure 3B). The average $K_{p,uu}$ from the coadministration group was found to be between 1.5 and 3, with no significant differences from either the free DPH or PEG liposomal DPH group (Table 1 and Figure 3B).

Individual plasma and brain concentration−time curves are depicted in Figure S1. For the free DPH group, the curves in plasma and brain were parallel, and brain concentrations in all animals showed active uptake (Figure S1A). It was clearly observed in 7 out of 8 animals in the liposomal DPH group that the unbound brain profile declined faster than in plasma in the early phase, and thereafter stabilized (Figure S1B). In the coadministration group, the unbound and total plasma curves deviated from each other from around 120 min after the start of infusion (Figure S1C).

DISCUSSION

When developing liposome-based strategies to improve drug delivery into the brain, the importance of understanding how the payload itself is transported at the BBB is often underrated. Having seen the positive impact of liposomes on the brain delivery of drugs with active efflux at the BBB,[5,6] one may assume that the liposomal encapsulation would increase the brain uptake of all drugs at the BBB, thus having more opportunities to achieve targeted delivery and improved effect. Our present study has shown that this wish may not be fulfilled.

This article shows that the brain uptake of DPH with active influx at the BBB is reduced after encapsulation in PEG-EYPC liposomes, with a significantly smaller $K_{p,uu}$ of 1.5 vs 3.0 when administering the free drug. A reduction in brain uptake was also observed in the study of quetiapine after nanoencapsulation.[8] Combined with our previous findings that certain types of PEG liposomes can improve the brain delivery of drugs with active efflux at the BBB,[5,6] it seems that liposomal encapsulation can influence BBB transport of drugs in different directions depending on how the drug interacts with the BBB. To be noted is that in our earlier study of MTX, only one of two liposomal formulations were able to increase brain uptake, indicating that in vivo delivery of drugs in liposomes is not a unified process.[5] For drugs with active influx ($K_{p,uu} > 1$), liposomal encapsulation does not seem to be a good solution if the purpose is to further increase the brain uptake, although examples are still sparse.[8] Therefore, it is of crucial importance to be aware of the basic BBB transport features of the drug intended to be encapsulated.

Interestingly, it seems that the coadministration of empty PEG liposomes together with free DPH can also decrease the brain uptake of DPH, although the reduction was smaller than that from PEG liposomal DPH, reflected by a $K_{p,uu}$ between 1.5 and 3.0. A similar free drug + empty liposomes group was also included in a previous study in order to examine if glutathione pegylated liposomes themselves increased brain delivery of DAMGO by influencing BBB function. The absence of difference in $K_{p,uu}$ compared with administering free DAMGO excluded this possibility.[6] However, the decrease in $K_{p,uu}$ from 3.0 to 2.3 observed here does not necessarily suggest that empty PEG liposomes themselves can actually alter BBB function by inhibiting influx transporters. In fact, when empty liposomes were administered simultaneously with free DPH, the decreased unbound fraction of DPH and deviation between unbound and total plasma PK curves during the later period indicate a potential drug binding to the liposomes. These liposomes may instead interact with the BBB in a similar way as the DPH-encapsulated liposomes, which may explain the reduction of $K_{p,uu}$ compared to free drug administration. Regardless of the effect on BBB transport, the DPH binding to PEG liposomes was in itself an interesting finding, indicating another possible in vivo drug and liposome interaction. This finding can only be observed with the current study design when administering both free drug and free drug + empty liposomes and with unbound and total plasma drug concentrations measured separately.

Combining the present results with earlier in vivo results of MTX and DAMGO, it could be speculated if liposomal encapsulation is able to hinder transporter function.[5,6] Both MTX and DAMGO have active efflux at the BBB, and their transport into the brain was improved when encapsulated into liposomes. For DPH with active uptake at the BBB, the opposite was found with a decreased uptake after encapsulation. This was also the case for quetiapine.[8] Thus, it seems as if transporter function is reduced independent of direction. However, when administering empty liposomes together with free DAMGO, no influence was observed on the brain uptake of DAMGO.[6] Therefore, more studies are required to further elucidate the intricate interactions among drugs, liposomes, and the BBB transporters responsible for active influx or efflux of drugs.

The presence of a biphasic PK profile of total DPH in plasma with a fast decline in the early period suggests that a part of the encapsulated DPH was released early in vivo, which correlates with the in vitro observations. The biexponential PK profile is quite different from the behavior of MTX in the same formulation.[5] However, this is not uncommon for PEG liposomal formulations, as reported in other studies.[16,17] Moreover, the terminal $t_{1/2}$ for PEG liposomal DPH (around 3.5 h) is still within the reported range of the half-lives of PEG liposomes (from 2 to 24 h).[18]

The $K_{p,uu}$ of DPH showed a dramatic decrease from 14 to 3 within the first hour after free DPH administration, followed by a relatively constant behavior thereafter. The average values of $K_{p,uu}$ in the two periods were actually similar to the results from a previous study where $K_{p,uu}$ was 5.5 during the constant infusion period, followed by a decrease to approximately 3 in the elimination phase after the infusion stopped.[9] Earlier in vitro results have suggested that DPH was actively transported into the brain in a rapid and saturable manner.[9,19] However, saturation of uptake might be expected to occur with increasing unbound concentration in plasma. What we observed in the two studies is the opposite, with rather a time-influence than a direct concentration influence. We do not at the present time have an explanation to this phenomenon.

During the first hour after administration of PEG liposomal DPH, a similar drastic decrease was observed for $K_{p,uu}$, with no early difference in the average $K_{p,uu}$ compared to the free drug group. However, after this period, the $K_{p,uu}$ decreased significantly more than after free DPH administration and to values that indicate dominating passive transport, i.e., values close to unity. We speculate that the absence of liposomal influence during the initial period may be associated with the

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early drug release from the liposomes. Therefore, the $K_{p,uu}$ during the second period may be a better indicator reflecting how DPH is transported at the BBB with and without liposomal encapsulation. This is because an equilibrium between unbound brain and plasma concentrations was reached during this period, indicated by a stable $K_{p,uu}$ in both groups. Therefore, on the basis of the $K_{p,uu}$ in the 60−360 min period, we conclude that PEG liposomal encapsulation reduced the brain uptake of DPH. Our findings emphasize the importance of understanding how the payload itself is transported at the BBB before designing and developing any nanocarrier-based strategy aiming at improving delivery of drugs into the CNS.

CONCLUSIONS

In the present study, we were able to provide in vivo quantitative evidence on how PEG liposomes influence the brain uptake of DPH by using microdialysis. Compared with free DPH administration, the encapsulation in PEG liposomes significantly reduced DPH uptake into the brain. The coadministration with empty PEG liposomes also showed a tendency to decrease the transport of DPH at the BBB. These results, together with previous findings on MTX and DAMGO, give a better fundamental understanding of how PEG liposomes influence the BBB transport of cargoes in different directions. Our observations suggest that the in vivo BBB transport property of the payload is a key factor affecting the outcome of liposomal brain delivery. Therefore, this needs to be considered in the early stage of design and development of liposomal strategies for the treatment of CNS diseases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.8b00611.

Individual concentration−time profiles of unbound DPH in brain (open triangles), plasma (open circles), and total DPH in plasma (filled circles) after 30 min i.v. infusion of 4.5 mg/kg of free DPH, PEG liposomal DPH, and free DPH + empty PEG liposomes (PDF)

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Notes

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