Supplementary information

Supplementary Methods

Synthesis of deacetyl-diltiazem and determination of pK_as

The DTZ metabolite, DAD, was obtained by deacetylation of 500 mg of diltiazem in 0.5 M NaOH in methanol/water 40% (*v/v*) under stirring at room temperature for 1 h. The product was recovered by liquid-liquid extraction with dichloromethane after evaporating the methanol under reduced atmosphere. The yield was ~65%, and structure was confirmed by H-NMR (^{1}H, 300 MHz, $CDCl_3$) δ (ppm) 2.31 (s; 6H), 2.51 (m; 1H), 2.74 (m; 1H), 3.74 (m; 1H), 3.85 (s; 3H), 4.32 (d; J= 4.0 Hz, 1H), 4.51 (m; 1H), 4.92 (d; J= 4.0 Hz; 1H), 6.92 (d; J= 4.0 Hz ; 2H), 7.11-7.53 (m, 5H), 7.73 (m; 1H), ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm) 46.39, 48.49, 56.12, 57.4, 70.1, 114.5, 125.3, 127.0, 128.5, 129.7, 131.5, 132.2, 136.2, 145.6, 160.7, 172.2, and high resolution mass spectrometry (on LC-MSD TOF (Agilent Technologies, Santa Clara, CA)) Calculated for $C_{20}H_{25}N_2O_3S$: 373.15804; Found 373.15745; δ= -1.58 ppm. Purity in excess of 97% was confirmed by HPLC.

The pK_as of DTZ and DAD were determined in 35 % methanol/water (*v/v*) by direct alkaline titration, and the pK_a in pure water ($^w_w pK_a$) was extrapolated with equation 1[1]:

$$^s_s pK_a = a_s \, ^w_w pK_a + b_s \quad (1)$$

Where $^s_s pKa$ is the pK_a obtained by titration in 35 % methanol, and values of a_s and b_s are 0.988, and -0.204 as proposed for 35 % methanol in [1].

Interaction of bromophenol blue (BPB) with DTZ

The interaction of 0.025% BPB with diltiazem was assessed by incubating the probe with different amounts of drug[2]. BPB-loaded liposomes were prepared by the lipid-film hydration/extrusion method using a pH 3, 200-mM citrate buffer containing 0.025% BPB. Immediately after SEC purification, liposomes were incubated in HBS pH 7.4 buffer with or without 1 mM DTZ. The BPB absorbance was measured from 350 to 800 nm with a Safire plate reader (Tecan, Durham, NC) after 10 and 30 min. At the end of the experiment, the liposomes were lysed with 10 µL of Triton® X-100 (10%).

Determination of captured volume of liposomes

The entrapped (or captured) volume of liposome (measured in µL/µmol of PL) corresponds to the aqueous volume delimited by the PL membrane[3]. It varies according to membrane lamellarity, PL concentration, and vesicle size distribution and shape[4]. To determine it experimentally, liposomes of different diameters were prepared by extrusion as described in the Methods section except that [^{14}C]-citric acid (100-124 mCi/mmol, Perkin Elmer, Waltham, MA) was added to the hydration buffer at a concentration of 0.5 µCi/mL. After purification by SEC on a Sephadex G-50 column, the

entrapped amount of [^{14}C]-citric acid was immediately measured by scintillation counting, and volume was normalized for PL content[4, 5]. The entrapped volume to diameter (Z-average) curve was fitted with descriptive empirical equation 2:

$$Entrapped\ volume = y_0 - A\ e^{(-B \times Diameter)} \quad (2)$$

A R^2 = 0.999 was obtained with values of 3.426, 2011.428, and 0.057 for y_0, A and B, respectively. This equation was then applied to quantify the entrapped volume of liposomes of different diameters prepared without [^{14}C]-citric acid, and investigated in uptake studies as described in Methods. Capture capacity *vs.* entrapped volume is plotted in Fig. S4.

Stability of transmembrane pH gradient

8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine) (Invitrogen, Carlsbad, CA) fluorescence served as a pH indicator to show stability of the pH gradient in liposomes after exposure to 80 % rat plasma. Liposomes were prepared as described in the Methods section, except that the citrate hydration buffer (pH 3, 200 mM) contained 5 mM pyranine. Liposomes were purified by SEC on a Sephadex G-50 column, and incubated in 80% plasma or HBS. After different incubation times (0, 1, 2 and 6 h), the liposomes were purified by SEC on a Sepharose CL-4B column. The fluorescence spectra of entrapped pyranine (from 350 to 495 nm) were measured in a 96-well plate, on a Safire plate reader (Tecan, Durham, NC) with an emission wavelength of 520 nm. The ratio of fluorescence at 405 over 455 nm was used to determine the increase in pH[6].

Pharmacokinetics of liposomes and encapsulated citrate buffer

Dual-labeled liposomes were prepared as described in the Methods section. [^{3}H]-cholesteryl hexadecylether ([^{3}H]-CHE) (50-60 mCi/mmol, American Radiolabeled Chemicals, St Louis, MO) and [^{14}C]-citric acid (100-124 mCi/mmol, Perkin Elmer, Waltham, MA), were added during lipid-film preparation and hydration, respectively. Liposomes were purified by dialysis (Spectra/Por regenerated cellulose with a membrane cut-off of 6-8000 kDa) and the blood profiles of both isotopes were monitored in Sprague-Dawley rats by scintillation counting as described elsewhere[7].

***In vitro* bupivacaine capture study.**

Bupivacaine capture by the transmembrane pH-gradient liposomes was determined by incubating 1 mM bupivacaine HCl (Sigma, St Louis, MO) spiked with radiolabeled [^{3}H]-bupivacaine HCl (1-5 Ci/mmol, Moravek, Brea, CA) with liposomes or IFE 20% (2.5 mM of PL) as described for the DTZ capture experiment. Free drug was separated from the colloidal formulation by SEC, and scintillation counting was used to determine the amount of drug in each fractions. Capture capacity was normalized for PL content.

Deacetylation of encapsulated DTZ in blood

Liposomes were prepared, and DTZ was loaded in vesicles by incubation as outlined in the Methods section. Free drug was removed from the vesicles by SEC, and liposomes containing 300 µg of DTZ and 10 µg of DAD were incubated in 1 mL HBS, pH 7.4, or

freshly collected, EDTA-anticoagulated rat blood (Ht ≈60%). At different time intervals, the DAD/DTZ ratio in solution (total) and in unbound form (free) was assessed by HPLC. The free drug concentration was obtained after ultrafiltration at 12,000 *g* on 30-kDa Amicon Ultra-0.5, centrifugation units (Millipore, Billerica, MA). The drug ratio inside the liposomes was determined by subtracting free DTZ and DAD concentrations from total concentrations in solution. At all time points, the percentage of leaked drug was below 12% of total content.

Supplementary secondary pharmacokinetic parameters

The area under the first moment curve from 0 to 6h ($AUMC_{(0\text{-}6h)}$) was calculated using the trapezoidal method. The area under the first moment curve from 6 h to infinite ($AUMC_{(6h\text{-}\infty)}$) was determined using equation 3:

$$AUMC_{(6h-\infty)} = \frac{C_{last} \times t_{last}}{K_{el}} \times \frac{C_{last}}{K_{el}} \quad (3)$$

The area under the first moment curve from 0 to infinite ($AUMC_{(0\text{-}\infty)}$) was obtained from the addition of $AUMC_{(0\text{-}6h)}$ and $AUMC_{(6h\text{-}\infty)}$. The mean residence time (MRT) was calculated from the ratio of $AUMC_{(0\text{-}\infty)}$ to $AUC_{(0\text{-}\infty)}$. The volume of distribution at steady-state (Vd_{ss}) was calculated by multiplying the MRT by Cl, and the volume of distribution based on the total area under the concentration *vs*. time curve (Vd_{area}) by dividing Cl by K_{el}.

Table S1. Liposome formulations tested. Mean ± SD (n=3).

	Internal buffer			Vesicle diameter		
	pH	Citrate conc. (mM)	Osmolarity (mOsm/kg)	Z-average (nm)	PdI	Notes
Figure 2A	**2**	150	298±1	130±4	0.074±0.014	-
	3	150	305±1	141±1	0.094±0.057	-
	4	150	295±1	150±3	0.067±0.016	-
	5	150	360±2	137±4	0.055±0.016	-
Figure 2B	3	**50**	73±2	139±5	0.068±0.019	-
	3	**100**	150±1	142±4	0.048±0.004	-
	3	**150**	220±1	141±2	0.054±0.009	-
	3	**175**	255±1	140±3	0.054±0.009	-
	3	**200**	293±1	146±2	0.054±0.012	-
	3	**225**	328±1	144±4	0.074±0.006	-
	3	**250**	365±2	141±3	0.047±0.004	-
Figure 3	3	200	293±1	143±3	0.067±0.018	**Optimized formulation**
	7.4	*NA*	292±1	143±4	0.069±0.009	***EPC/Chol containing HBS, pH 7.4***
	7.4	*NA*	292±1	143±2	0.062±0.015	***DMPG/Chol containing HBS, pH 7.4***
	-	-	-	285±6	0.121±0.015	***Intralipid® 20%***
Figures 4 & 5	3	200	293±1	141±4	0.093±0.004	*Fig. 4*
	3	200	293±1	142±2	0.068±0.015	*Fig. 5A*
	3	200	293±1	145±3	0.041±0.027	*Fig. 5B*
Figure S2	3	50	**73±2**	139±5	0.068±0.019	-
	3	100	**150±1**	142±4	0.048±0.004	-
	3	150	**220±1**	141±2	0.054±0.009	-
	3	50	**296±1**	139±5	0.054±0.008	
	3	100	**302±2**	139±4	0.053±0.002	*Made iso-osmolar with NaCl*
	3	150	**305±1**	144±4	0.049±0.012	

Table S1 (cont'd)

Figure S3	**3**	200	343±3	157±3	0.061±0.015	Containing 0.025% bromophenol blue (BPB)
Figure S4	4	150	295±1	**128±3**	0.065±0.017	*Containing [^{14}C]-citrate for internal volume measurement*
	4	150	295±1	**144±2**	0.087±0.012	
	4	150	295±1	**170±6**	0.056±0.014	
	4	150	295±1	**190±4**	0.163±0.028	
	4	150	295±1	**127±3**	0.080±0.006	-
	4	150	295±1	**153±2**	0.078±0.007	-
	4	150	295±1	**159±2**	0.066±0.026	-
	4	150	295±1	**174±1**	0.120±0.030	-
	3	150	293±1	**181±6**	0.079±0.011	-
	3	150	293±1	**142±3**	0.047±0.030	-
	3	150	293±1	**127±2**	0.051±0.014	-
Figure S5	3	200	320±3	132±4	0.051±0.013	*Containing 5 mM pyranine*
	3	200	320±3	156±1	0.071±0.016	
	3	200	320±3	158±2	0.067±0.010	
Figure S6	3	100	150±1	135±4	0.049±0.026	*Containing [^{3}H]-CHE and [^{14}C]-citric acid*
Figure S7	3	200		148±3	0.056±0.030	-
	-	-	-	285±6	0.121±0.015	*Intralipid® 20%*
Figures S8 & S9	3	200	293±1	148±5	0.037±0.022	*Fig. S5*
	3	200	293±1	142±2	0.099±0.012	*Fig. S6 Loaded with DTZ before incubation*

PdI: Polydispersity index

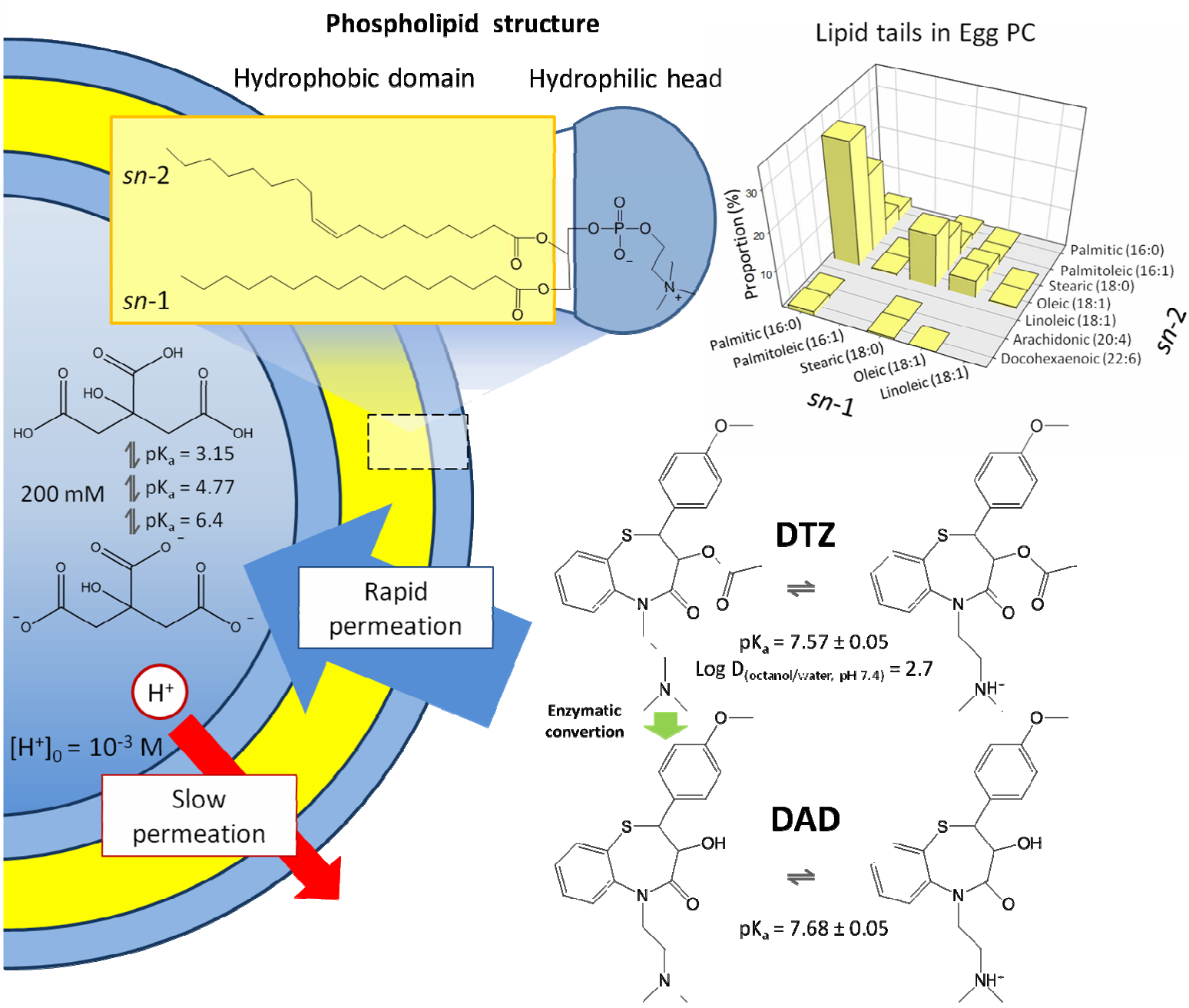

Figure S1. Structures of Egg PC, citrate, DTZ and DAD. Egg PC lipid chains were obtained from reference[8] and DTZ partition coefficient from reference[9]. $[H^+]_0$: initial proton concentration.

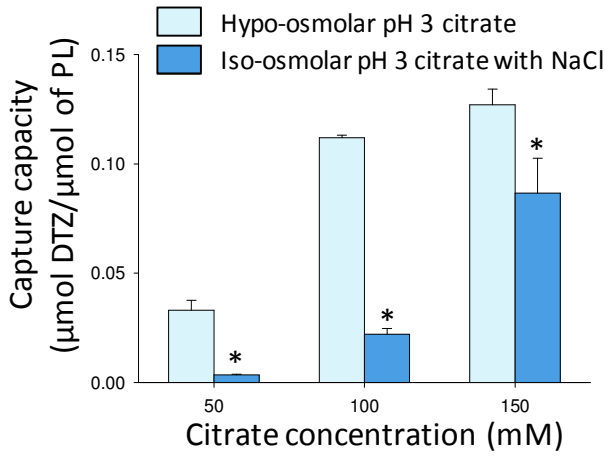

Figure S2. Influence of ionic strength on capture. Mean ± SD (n=3), *p<0.05 *vs.* same citrate concentration hypo-osmolar buffer. With liposomes containing the same citrate concentration, buffers made iso-osmolar with NaCl are less efficient to take up DTZ than their hypo-osmolar counterpart. Ion concentration inside the liposomes affects drug capture.

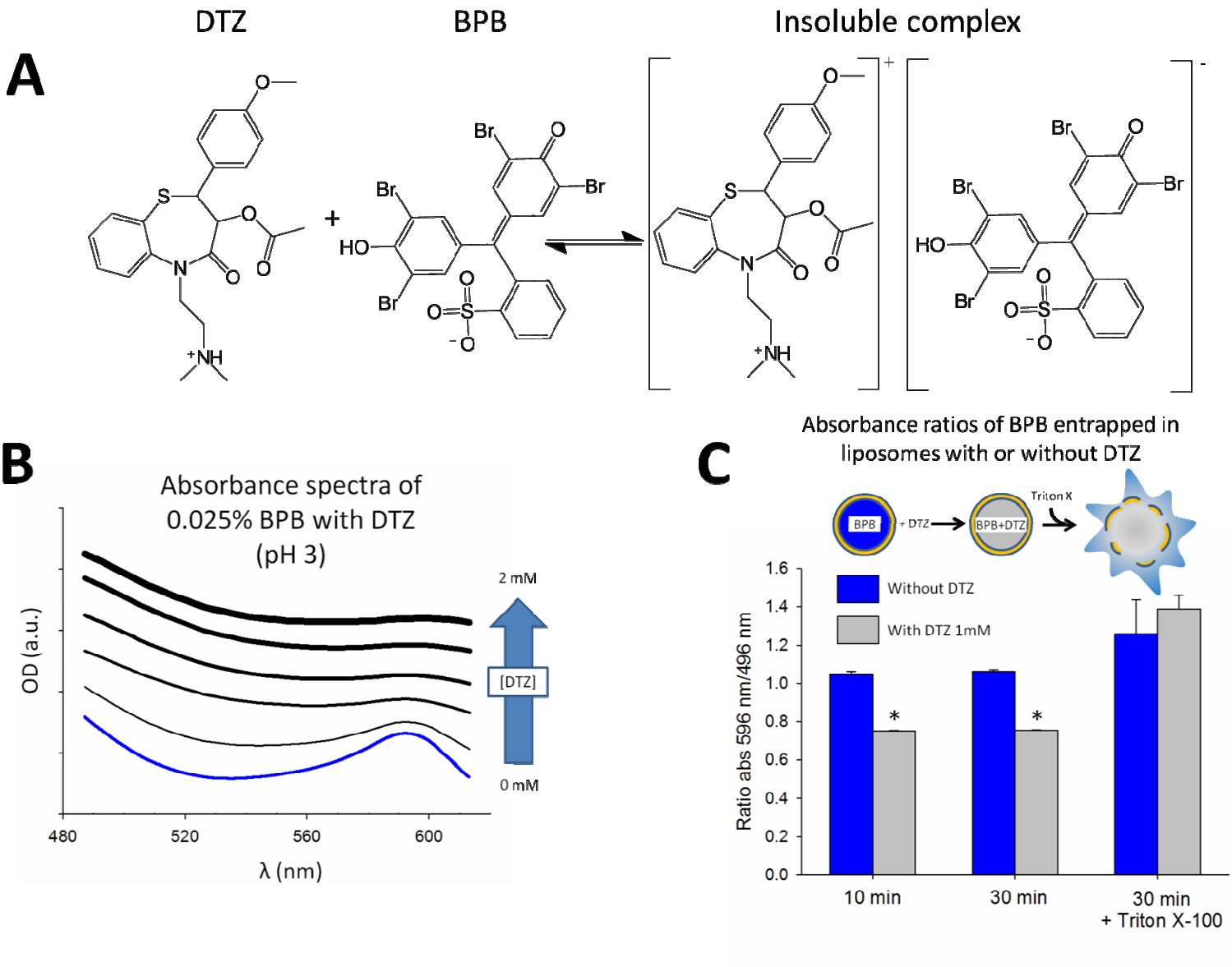

Figure S3. Interactions of DTZ with BPB inside the liposomes. **(A).** Schematic representation of the interaction between DTZ and BPB. **(B).** Absorbance spectra of BPB with increasing DTZ concentrations. **(C).** Ratio of absorbance (596 nm/496 nm) of BPB entrapped in liposomes with or without DTZ 1 mM (Mean ± SD, n=4) * $p<0.05$ *vs.* incubation without DTZ. Interactions between the drug and the encapsulated probe show that DTZ is rapidly pumped inside the liposomes as a result of the transmembrane pH gradient. Solubilization of the liposomes with Triton® X-100 disrupts the interactions.

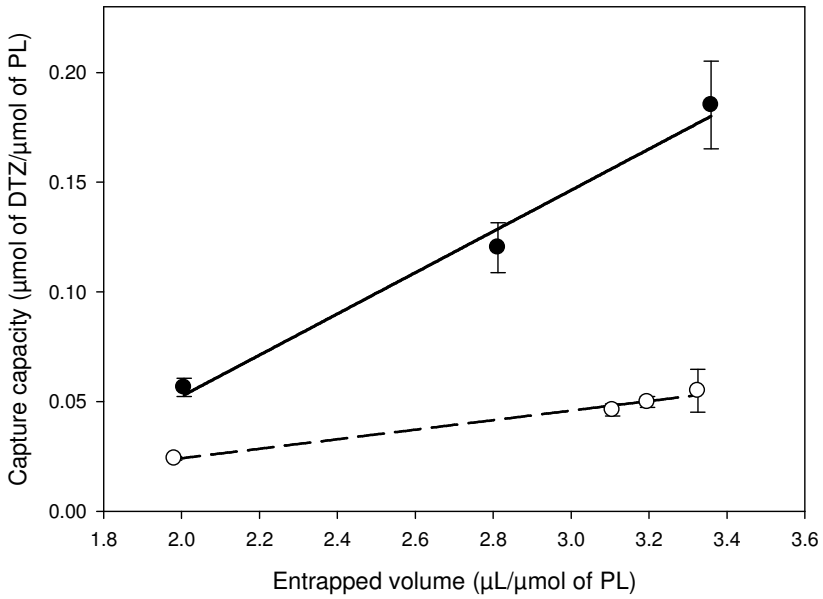

Figure S4. Influence of entrapped volume in vesicles on DTZ uptake capacity. Full and empty circles represent vesicles with internal pH 3.0 and 4.0, respectively. Mean ± SD (n=3). Capture efficiency increases with the volume entrapped in vesicles.

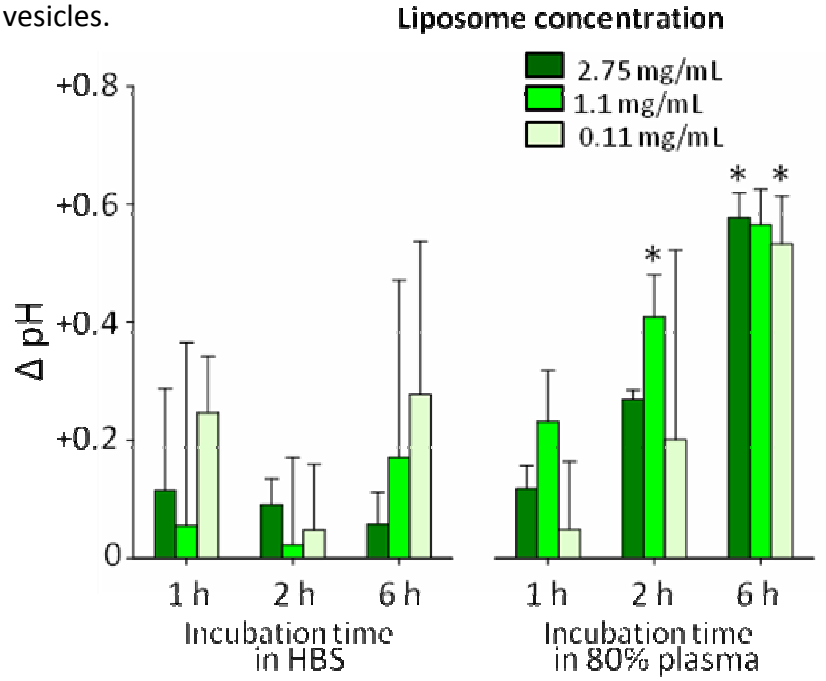

Figure S5. Stability of pH gradient in the presence of plasma. Mean ± SD (n=3-5), *p<0.05 *vs.* same incubation time in HBS. The pH gradient remains stable when incubated in HBS and increases by less than 0.6 pH units over 6 h when incubated in 80% plasma. Since the liposomes concentrations used in this experiments are comparable to those which could be achieved after

intravenous injection, it can be concluded that the pH-gradient should remain stable under physiological conditions.

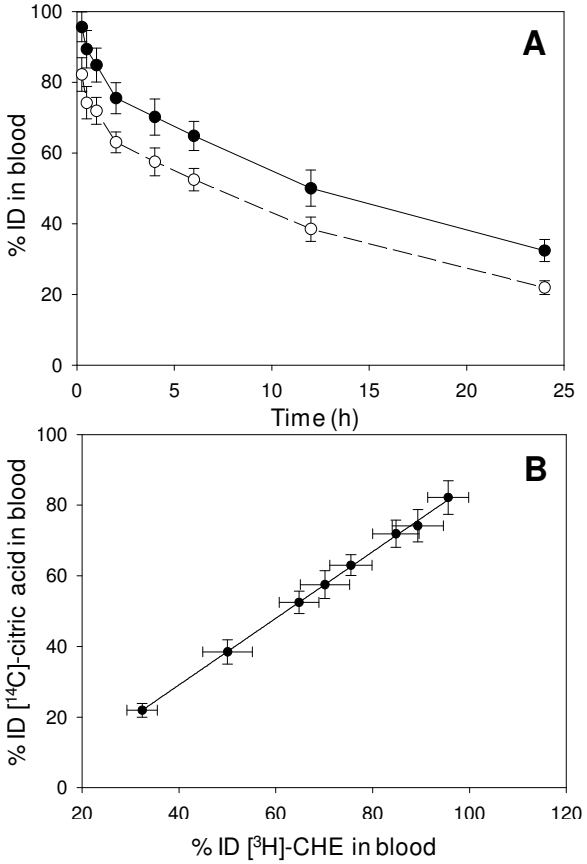

Figure S6. Stability of citric acid entrapment in blood. (**A**) Blood circulation profiles of [^{3}H]-CHE-labelled transmembrane pH-gradient liposomes (full circles) and encapsulated [^{14}C]-citrate buffer (empty circles). (**B**) Linear relationship between injected doses of vesicles and citrate. Mean ± SD (n=6). The parallel circulation profiles of both isotopes, as well as the linear relation between the circulating doses of vesicles and entrapped citrate buffer, indicate that minimal citrate leakage occurs in the bloodstream during the first 24 h.

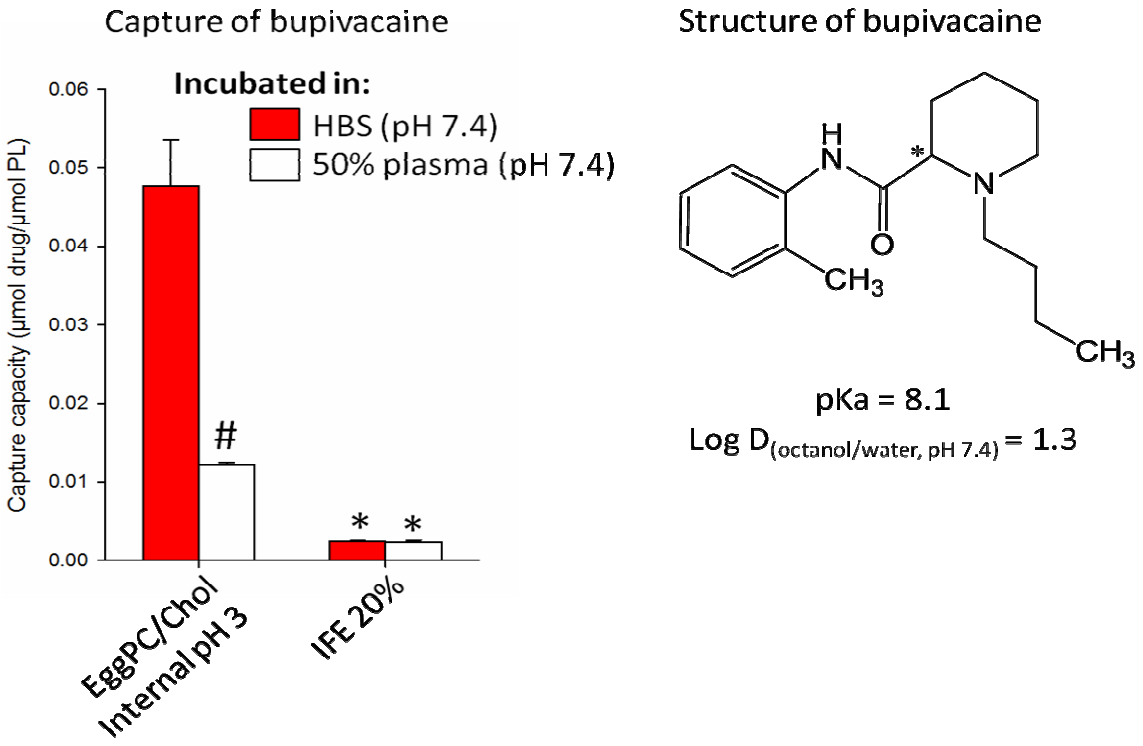

Figure S7. Capture of bupivacaine by liposomes with transmembrane pH-gradient and IFE 20%. (Mean ± SD, n=3). # $p<0.05$ *vs.* same formulation in HBS, * $p<0.05$ *vs.* liposomes EggPC/Chol internal pH 3 in same incubation medium. Despite the physicochemical differences *vs.* DTZ (pK_a and log D obtained from references[10, 11]), the liposomes can efficiently capture bupivacaine.

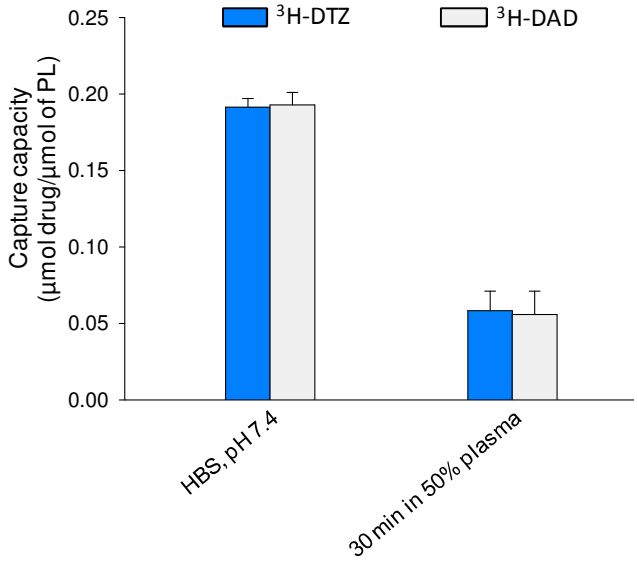

Figure S8. *In vitro* capture of DTZ and DAD. Mean ± SD (n=3), *p<0.05 *vs.* DTZ in same conditions. Both DTZ and DAD are taken up to a similar extent *in vitro* in the presence and absence of 50% rat plasma. Incubation time in plasma was set at 30 min to minimize the *in situ* conversion of DTZ to DAD. The pK_as of DTZ and DAD are 7.57 ± 0.05 and 7.68 ± 0.05, respectively.

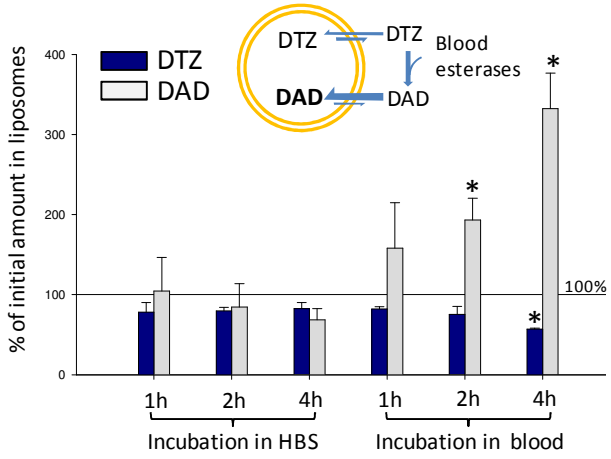

Figure S9. Percentage of initial DTZ and DAD amounts in liposomes after incubation in HBS and blood. Mean ± SD (n=3), *p<0.05 *vs*. the same drug (DTZ or DAD) after the same incubation time in HBS. The quantities of DTZ and DAD in liposomes remain stable in HBS while the amount of DTZ present in liposomes incubated in blood decreases with the conversion of DTZ to DAD caused by esterases.

Table S2. Supplementary secondary PK parameters. Mean ± SD (n =7-8), *p<0.05 *vs.* control.

		Control	Liposomes
DTZ	K_{el} (h^{-1})	0.97 ± 0.36	0.85 ± 0.28
	$AUC_{(0-\infty)}$ (ng.h/mL)	1562 ± 458	9 298 ± 1637*
	MRT (h)[1]	0.92 ± 0.28	0.29 ± 0.13*
	AUMC (ng.h^2/mL)	1479 ± 846	2617 ± 1040*
	Vd_{ss} (mL/kg)	3023 ± 1123	162 ± 93*
	Vd_{area} (mL/kg)	3795 ± 1274	725 ± 283*
DAD	K_{el} (h^{-1})	1.26 ± 0.28	1.52 ± 0.85
	$AUC_{(0-\infty)}$ (ng.h/mL)	1337 ± 582	31,364 ± 13,418*
	MRT (h)	0.70 ± 0.32	0.86 ± 0.24
	AUMC (ng.h^2/mL)	1050 ± 1050	29,518 ± 19,842*

[1]The lower MRT for DTZ in the liposome group could be explained by the change in metabolic profile and V_d of the encapsulated drug (see Table 1 and Fig. S9).

Table S3. Hemodynamic parameters after DTZ injection in the presence and absence of liposomes. Mean ± SD (n =5-6), *p<0.05 *vs.* control.

		Diastolic		Systolic		MAP	
		Control (NS)	Liposomes (275 mg/kg)	Control (NS)	Liposomes (275 mg/kg)	Control (NS)	Liposomes (275 mg/kg)
DTZ Bolus (5 mg/kg)	**Δ Max** (%)	-53 ± 12.3	-40 ± 3.4*	-46 ± 8.7	-34 ± 3.9*	-50 ± 10.7	-38 ± 3.4*
	$AUC_{(0-60min)}$ (%.min)	1484 ± 508	964 ± 221*	1003 ± 184	670 ± 225*	1287 ± 362	853 ± 203*
DTZ Bolus (5 mg/kg) + **Perfusion** (8 mg/kg/h)	**Δ Max** (%)	-57 ± 3.4	-42 ± 4.4*	-54 ± 3.1	-31 ± 3.0*	-56 ± 2.9	-37 ± 2.0*
	$AUC_{(0-60min)}$ (%.min)	2100 ± 394	1442 ± 140*	1716 ± 250	735 ± 188*	1929 ± 314	1149 ± 128*

Table S4. Statistical tests used in figures and tables.

	Number of samples	Reference	Symbol	Statistical test
Fig 3	n = 3	*vs.* same formulation in HBS	#	*t*-test
	n = 3	*vs.* EPC/Chol Internal pH 3 in same medium	*	Kruskal-Wallis and SNK
Fig S2	n = 3	vs. same citrate concentration hypo-osmolar	*	*t*-test
Fig S3	n = 4	*vs.* incubation without DTZ	*	Mann-Whitney U
Fig S5	n = 3-5	*vs.* same incubation time in HBS	*	*t*-test
Fig S7	n = 3	vs. same formulation in HBS	#	*t*-test
	n = 3	*vs.* EPC/Chol Internal pH 3 in same medium	*	*t*-test
Fig S8	n = 3	*vs.* DTZ in the same condition	*	*t*-test
Fig S9	n = 3	*vs.* same drug at same incubation time in HBS	*	*t*-test
Table 1	n = 7-8	*vs.* control	*	Mann-Whitney U
Table S2	n = 7-8	*vs.* control	*	Mann-Whitney U
Table S3	n = 5-6	*vs.* control	*	*t*-test

SNK: Student-Neuman-Keuls *post-hoc* test.

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