

Ultra-slow release of hydrophilic drugs via multilamellar–multivesicular liposomes formed by unsaturated phospholipids

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I. Supplementary Tables

Supplementary Table 1. Liposome formulations

Liposome	Molar ratio of lipids	Phospholipid molar percentage
L-DSPC[0]-Drug	DSPC : cholesterol = 15: 6	71%
L-SOPC[1]-Drug	SOPC : cholesterol = 15: 6	71 %
L-SLPC[2]-Drug	SLPC : cholesterol = 15: 6	71 %
L-DOPC[2]-Drug	DOPC : cholesterol = 15: 6	71 %
L-DSPC/DOPC[0/2]-Drug	DSPC : DOPC : cholesterol = 7: 8: 6	71 %
L-DLPC[4]-Drug	DLPC : cholesterol = 15: 6	71 %
L-DOPG[2]-Drug	DOPG : cholesterol = 15: 6	71 %

Nomenclature of liposomes: Abbreviations for liposomes follow the format: L-Lipid [#DB]-Drug. For example, liposomes with DOPC[2] loaded with TTX are abbreviated L-DOPC[2]-TTX. Liposomes with a mixture of DSPC[0] and DOPC[2] (DSPC: DOPC = 7: 8, mol%DOPC = 53 mol% of the phospholipids) loaded with TTX are abbreviated L-DSPC/DOPC[0/2]-TTX.

Supplementary Table 2. Physicochemical properties of liposomes

Liposomes ^a	Diameter (μm)	Zeta potential (mV)	TTX EE ^b (%)	TTX DL ^c (%)
L-DSPC[0]-TTX	4.1 ± 0.42	-0.94 ± 0.79	23 ± 5.5	0.12 ± 0.03
L-SOPC[1]-TTX	2.0 ± 0.26	-1.2 ± 0.69	44 ± 7.2	0.22 ± 0.04
L-SLPC[2]-TTX	1.7 ± 0.33	-1.5 ± 1.0	49 ± 2.9	0.24 ± 0.01
L-DOPC[2]-TTX	1.4 ± 0.07	-1.3 ± 1.1	72 ± 8.6	0.37 ± 0.04
L-DSPC/DOPC[0/2]- TTX	2.0 ± 0.10	-1.3 ± 0.57	32 ± 2.5	0.16 ± 0.01
L-DLPC[4]-TTX	1.2 ± 0.21	-1.7 ± 0.63	61 ± 3.0	0.31 ± 0.02
L-DOPG[2]-TTX	0.90 ± 0.06	-47 ± 2.0	42 ± 2.0	0.21 ± 0.01

Data are means $\pm$ SD, n = 4 independent experiments.

^aAbbreviations are explained in the main text.

^bEE: encapsulation efficiency

^cDL: drug loading

Supplementary Table 3. Sensory D-50 vs sensory D-tot

Formulation ^a	Drug and Dose	Sensory D-50 ^b (days)	Sensory D-tot ^c (days)	<i>P</i> values*	Difference ^d (%)
L-DSPC[0]	TTX 70 µg	0.04 ± 0.03	0.30 ± 0.24	<i>P</i> = 0.08	590%
	TTX 110 µg	0.17 ± 0.12	2.2 ± 2.5	<i>P</i> = 0.15	1220%
L-DOPC[2]	TTX 80 µg	6.5 ± 0.4	11 ± 1.1	<i>P</i> = 0.0004	64%
	TTX 120 µg	8.2 ± 1.0	13 ± 2.4	<i>P</i> = 0.01	57%
	TTX 160 µg	9.2 ± 1.4	14 ± 1.0	<i>P</i> = 0.001	52%
	TTX 200 µg	11 ± 0.7	15 ± 1.7	<i>P</i> = 0.004	39%
	TTX 260 µg	13 ± 1.0	18 ± 2.0	<i>P</i> = 0.004	40%
	TTX 100 µg & BUP 120 µg	8.4 ± 0.5	11 ± 1.1	<i>P</i> = 0.005	31%

Data are means ± SD, n = 4 rats per group.

^aAbbreviations are explained in the main text.

^b**D-50**: the time for latency to return to half-maximal (i.e., a latency of 7 s).

^c**D-tot**: the time for latency to return to baseline latency (i.e., a latency of 2 s).

^dDifference (%) between sensory D-50 and D-tot.

**P* values are from unpaired two-tailed t test to compare the sensory D-50 and D-tot.

Supplementary Table 4. Sensory D-50 vs motor D-50

Formulation ^a	Drug and Dose	Sensory D-50 ^b (days)	Motor D-50 ^c (days)	<i>P</i> values*
L-DSPC[0]	TTX 70 µg	0.04 ± 0.03	0.06 ± 0.04	<i>P</i> = 0.56
	TTX 110 µg	0.17 ± 0.12	0.10 ± 0.08	<i>P</i> = 0.43
L-DOPC[2]	TTX 80 µg	6.5 ± 0.4	6.7 ± 0.3	<i>P</i> = 0.39
	TTX 120 µg	8.2 ± 1.0	8.1 ± 0.9	<i>P</i> = 0.86
	TTX 160 µg	9.2 ± 1.4	8.8 ± 1.7	<i>P</i> = 0.74
	TTX 200 µg	11 ± 0.7	11 ± 1.0	<i>P</i> = 0.51
	TTX 260 µg	13 ± 1.0	14 ± 0.8	<i>P</i> = 0.07
	TTX 100 µg & BUP 120 µg	8.4 ± 0.5	8.7 ± 0.7	<i>P</i> = 0.37

Data are means ± SD, n = 4 rats per group.

^aAbbreviations are explained in the main text.

^b**Sensory D-50**: the time for latency to return to half-maximal (i.e., a latency of 7 s).

^c**Motor D-50**: the time for motor strength to return to half-maximal strength.

**P*-values are from unpaired two-tailed t test to compare the sensory D-50 and motor D-50.

Supplementary Table 5. Sensory D-tot vs motor D-tot

Formulation ^a	Drug and Dose	Sensory D-tot ^b (days)	Motor D-tot ^c (days)	<i>P</i> value*	Difference (%) ^d
L-DSPC[0]	TTX 70 µg	0.30 ± 0.24	0.06 ± 0.04	<i>P</i> = 0.092	
	TTX 110 µg	2.2 ± 2.5	0.16 ± 0.09	<i>P</i> = 0.15	
L-DOPC[2]	TTX 80 µg	11 ± 1.1	7.6 ± 0.4	<i>P</i> = 0.002	40
	TTX 120 µg	13 ± 2.4	8.9 ± 1.0	<i>P</i> = 0.02	46
	TTX 160 µg	14 ± 1.0	9.8 ± 1.8	<i>P</i> = 0.006	42
	TTX 200 µg	15 ± 1.7	13 ± 1.2	<i>P</i> = 0.065	
	TTX 260 µg	18 ± 2.0	16 ± 0.4	<i>P</i> = 0.24	
	TTX 100 µg & BUP 120 µg	11 ± 1.1	9.3 ± 0.5	<i>P</i> = 0.029	19

Data are means ± SD, n = 4 rats per group.

^aAbbreviations are explained in the main text.

^b**Sensory D-tot**: the time for latency to return to baseline latency (i.e., a latency of 2 s).

^c**Motor D-tot**: the time for motor strength to return to maximum strength before injection.

^dDifference (%) between sensory D-tot and motor D-tot, for those with a statistically significant difference.

**P* values are from unpaired two-tailed t test to compare sensory D-tot and motor D-tot.

Supplementary Table 6. Inflammation and myotoxicity after injection of formulations.

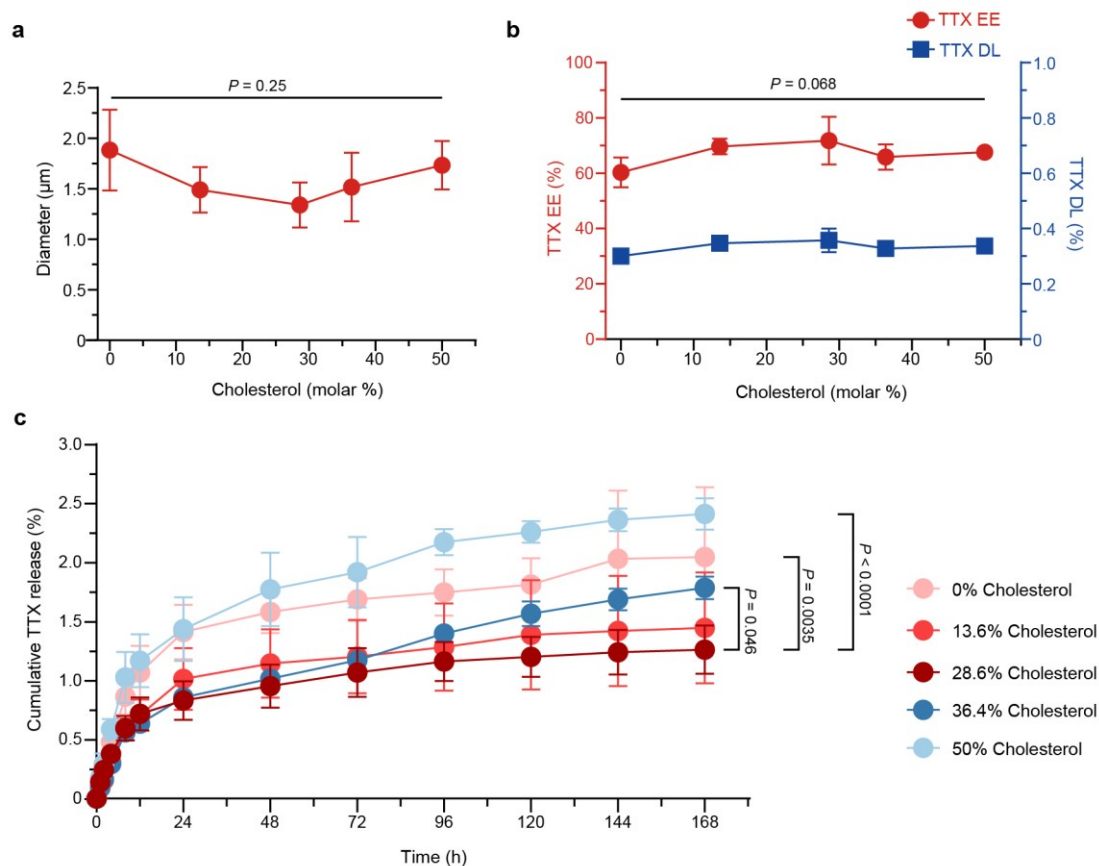
Formulation ^a	Inflammation scores		Myotoxicity scores	
Untreated	0 (0-0)		0 (0-0)	
	Day 4	<i>P</i> value*	Day 4	<i>P</i> value*
L-DSPC[0]-TTX	3.5 (2-4)	<i>P</i> < 0.05	0 (0-3)	<i>P</i> > 0.05
L-DOPC[2]-TTX	2 (1-2)	<i>P</i> < 0.05	1.5 (0-3)	<i>P</i> > 0.05
L-DOPC[2]-TTX-BUP	1.5 (1-3)	<i>P</i> < 0.05	1 (0-3)	<i>P</i> > 0.05
	Day 28	<i>P</i> value*	Day 28	<i>P</i> value*
L-DSPC[0]-TTX	0.5 (0-1)	<i>P</i> > 0.05	0 (0-0)	<i>P</i> > 0.05
L-DOPC[2]-TTX	0.5 (0-1)	<i>P</i> > 0.05	0 (0-0)	<i>P</i> > 0.05
L-DOPC[2]-TTX-BUP	1 (1-1.5)	<i>P</i> > 0.05	0.75 (0-1.75)	<i>P</i> > 0.05

Inflammation scores: 0 – 4. Myotoxicity scores: 0 – 6. Data are medians with 25th and 75th percentiles in parentheses; n = 4 rats per group.

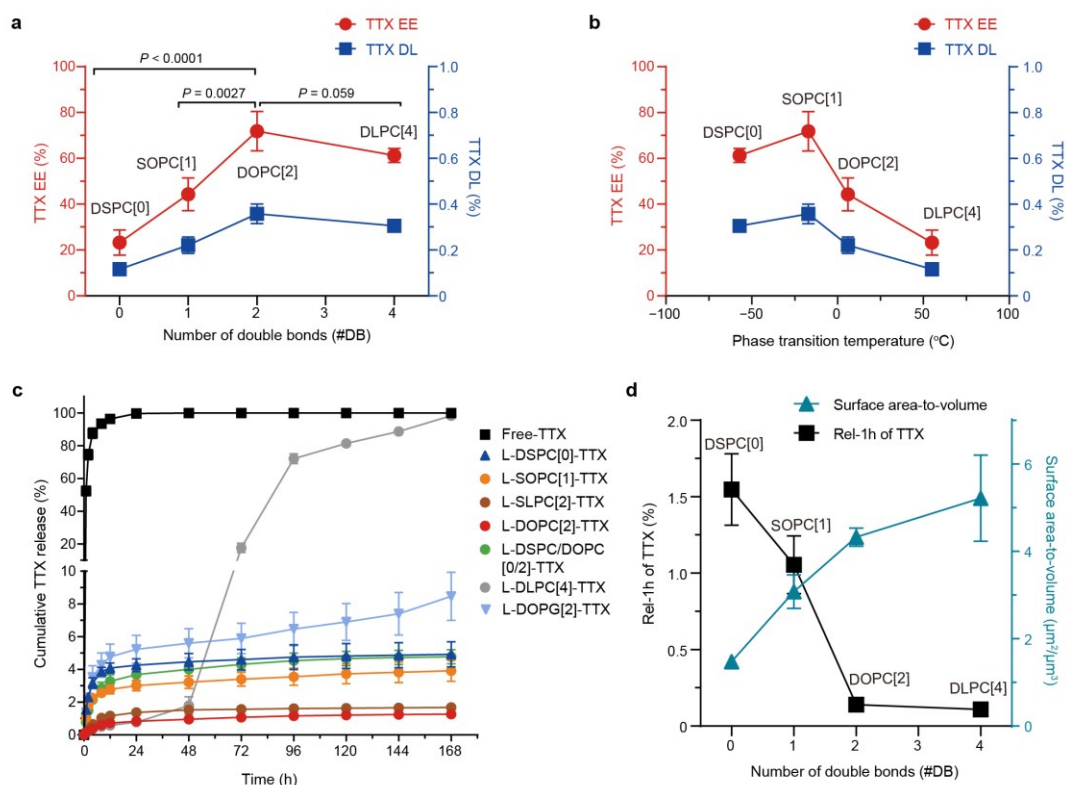
^aAbbreviations are explained in the main text.

**P* values are from the comparison of liposomal formulations to the untreated group, by Mann-Whitney U-test.

II. Supplementary Figures



Supplementary Fig. 1. Effect of cholesterol molar % on L-DOPC[2]-TTX. **a**, Effect of cholesterol content on liposome size, measured by dynamic light scattering ($n = 3$). P values are from one-way ANOVA. **b**, Effect of cholesterol content on encapsulation efficiency (EE) and drug loading (DL) of TTX ($n = 4$). Varying the cholesterol content had no statistically significant effect on liposome size, EE or DL. P values are from one-way ANOVA. **c**, Cumulative release of TTX from L-DOPC[2]-TTX with different cholesterol content ($n = 4$). P values compare groups at 168 h with an unpaired two-tailed t test. Release at 7 days (Rel-7d) from L-DOPC[2]-TTX with 28.6% cholesterol ($1.3 \pm 0.20\%$) was ~ 2 -fold less than with 50% cholesterol ($2.4 \pm 0.13\%$, $P < 0.0001$). Data are means $\pm$ SD. P values are from unpaired two-tailed t test.



Supplementary Fig. 2. Characterization of TTX-loaded liposomes with different #DB. **a**, Effect of #DB on EE and DL of TTX. For EE and DL, $r = 1$ for #DB 0 – 2. **b**, Effect of phospholipid T_m on EE and DL of TTX. For EE and DL, $r = -0.96$ for T_m in the range $-17\text{ }^{\circ}\text{C}$ to $55\text{ }^{\circ}\text{C}$. **c**, Cumulative release of TTX from different formulations in PBS at $37\text{ }^{\circ}\text{C}$. **d**, Relationship between Rel-1h of TTX, #DB and surface area-to-volume ratio in different liposome formulations. Data are means $\pm$ SD, $n = 4$ independent experiments. P values are from unpaired two-tailed t tests.

#DB: number of double bonds in phospholipids.

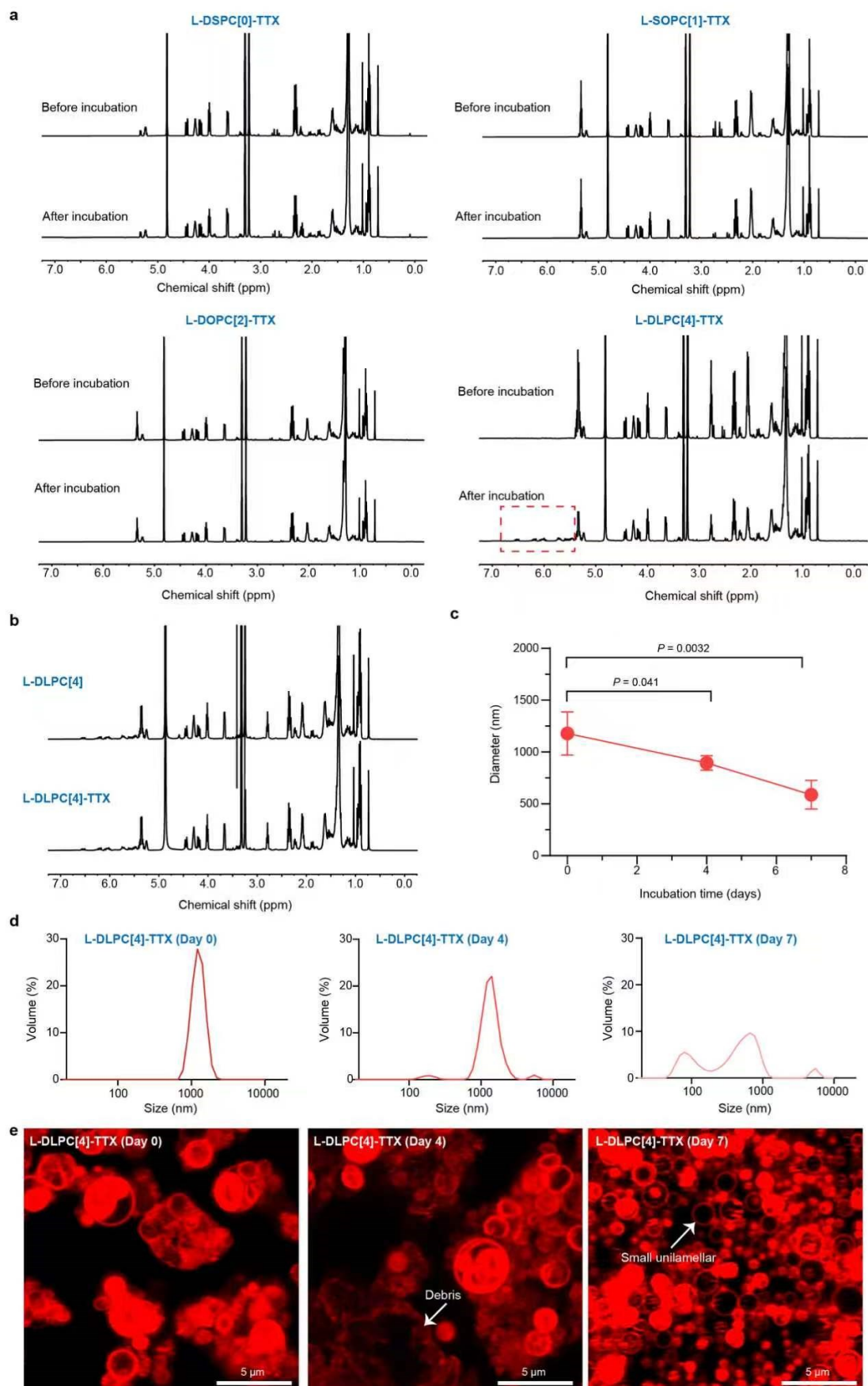
T_m : phase transition temperature.

Rel-1h: release at 1 h.

EE: encapsulation efficiency.

DL: drug loading.

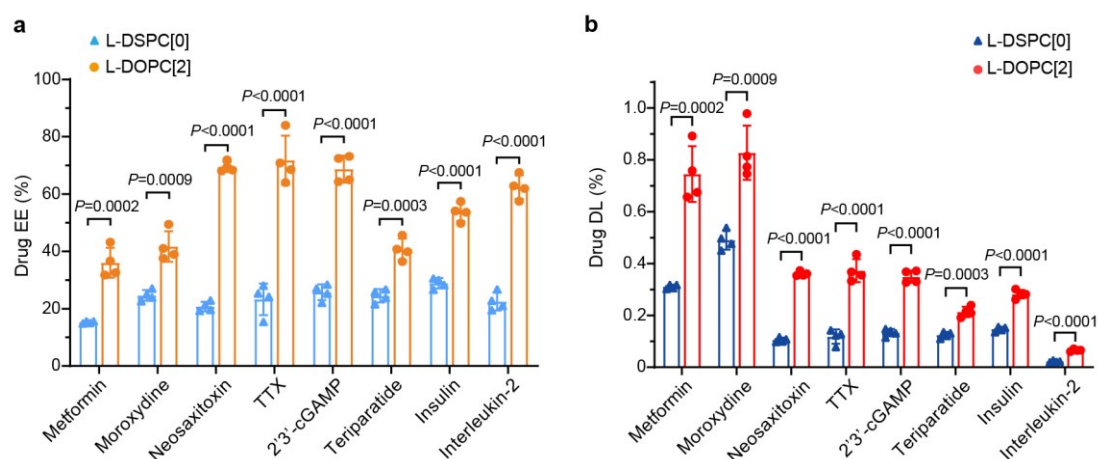
Liposome formulations are abbreviated as described in the main text.



Supplementary Fig. 3. Degradation of liposomes over 4 days at 37 °C in PBS.

a, ^1H -NMR spectra of liposomes, freeze-dried and redissolved in deuterated methanol, before or after incubation in release medium for 4 days. Peaks ($5.47 - 6.58 \text{ ppm}^1$) in the red dashed box correspond to primary oxidation products of DLPC[4], specifically conjugated dienes formed through the rearrangement of double bonds. **b**, ^1H -NMR spectra of L-DLPC[4] and L-DLPC[4]-TTX, incubated in release medium for 4 days, then freeze-dried and redissolved in deuterated methanol. **c**, Mean particle size over time of L-DLPC[4]-TTX. **d**, Size measurements by dynamic light scattering of L-DLPC[4]-TTX over time showing development of larger and smaller products. **e**, Representative (of 4 independent samples) confocal laser scanning microscopy of L-DLPC[4]-TTX with lipids stained with Nile Red on days 0, 4, and 7. Liposomal debris and small unilamellar liposomes were seen (arrows). Scale bar: $5 \mu\text{m}$. Data are means $\pm$ SD, $n = 4$ independent experiments. P values are from unpaired two-tailed t test.

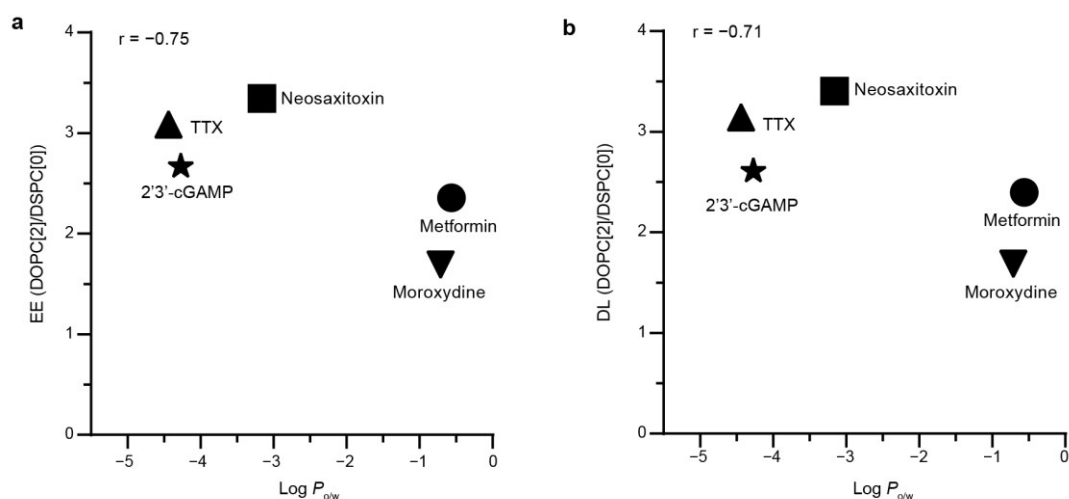
Lipid oxidation was confirmed by the detection of primary oxidation products (conjugated dienes, corresponding to peaks at $5.64 - 6.58 \text{ ppm}^1$) in the NMR spectra of L-DLPC[4]-TTX dissolved in deuterated methanol after 4 days incubation in release medium (Supplementary **Fig. 3a**). In contrast, L-DOPC[2]-TTX and L-DSPC[0]-TTX under identical conditions showed no detectable oxidation by NMR spectroscopy. Comparable conjugated-diene peaks were observed for L-DLPC[4] incubated under identical conditions in the absence of TTX (Supplementary **Fig. 3b**), indicating that oxidation occurs independently of drug. Dynamic light scattering measurements showed a 2-fold reduction in liposome size over 7 days (Supplementary **Fig. 3c, d**, $P = 0.0032$ for comparison of sizes on day 0 vs day 7). Confocal imaging of L-DLPC[4]-TTX at 7 days showed liposomal debris (Supplementary **Fig. 3e**).



Supplementary Fig. 4. Effect of #DB on the EE and DL of hydrophilic drugs. a, b, EE (a) and DL (b) in L-DSPC[0] and L-DOPC[2]. Data are means $\pm$ SD, $n = 4$ independent experiments. P values are from unpaired two-tailed t test.

EE: encapsulation efficiency.

DL: drug loading.

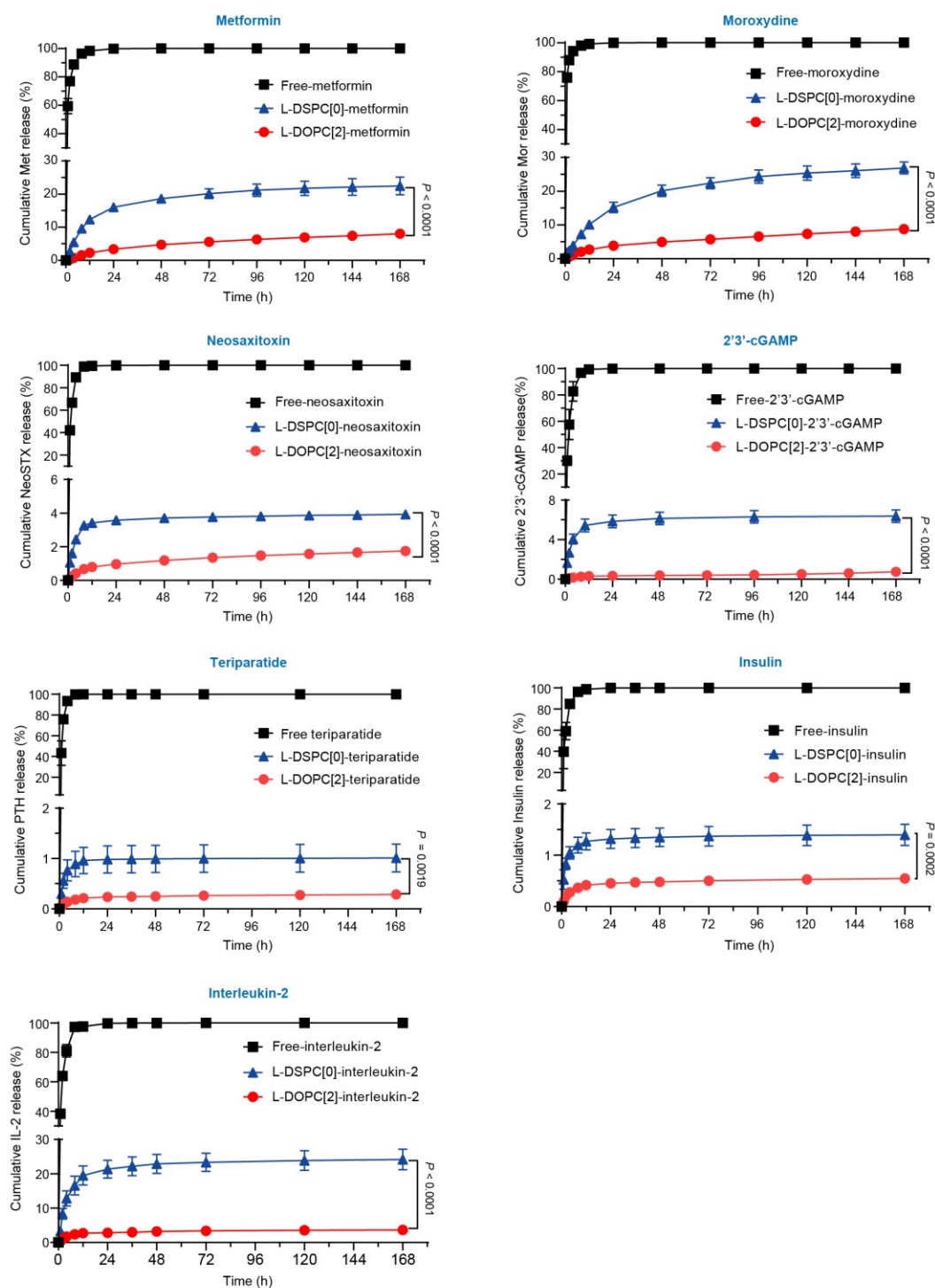


Supplementary Fig. 5. Effect of $\text{Log } P_{o/w}$ of hydrophilic drugs on EE and DL from using L-DOPC[2] vs L-DSPC[0]. a, b, EE (a) and DL (b) (DOPC[2]/DSPC[0]) as a function of $\text{Log } P_{o/w}$. Data are means $\pm$ SD, $n = 4$ independent experiments.

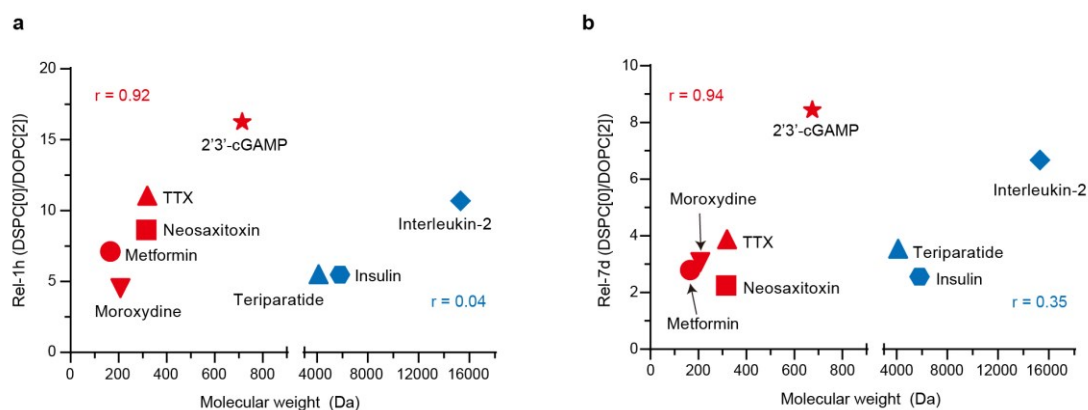
EE: encapsulation efficiency.

DL: drug loading.

Log $P_{o/w}$: logarithm of the octanol/water partition coefficient.



Supplementary Fig. 6. Cumulative release (%) of hydrophilic drugs from L-DSPC[0] and L-DOPC[2]. Data are means $\pm$ SD, $n = 4$ independent experiments. P values compare groups at 168 h with an unpaired two-tailed t test.

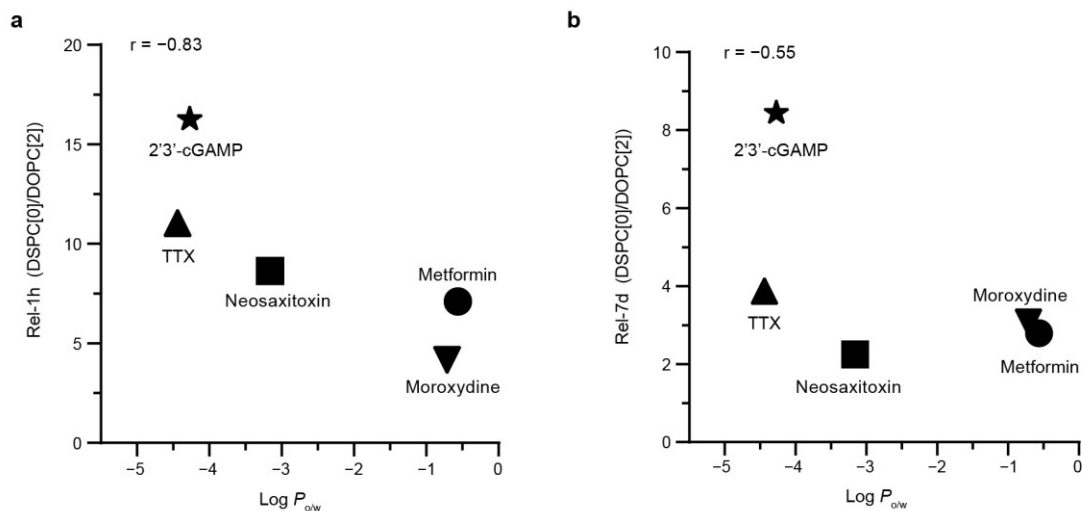


Supplementary Fig. 7. Effect of drug molecular weight on the reduction in release rate from using L-DOPC[2] vs L-DSPC[0]. The fold change is calculated as (release from L-DSPC[0]) / (release from L-DOPC[2]); values > 1 indicate slower release from L-DOPC[2]. **a**, Rel-1h. **b**, Rel-7d. Pearson correlation coefficients (r) are shown in blue for all tested molecules and in red for small molecules (< 1000 Da).

L-DOPC[2] significantly decreased Rel-1h, showing only 0.12 ± 0.02 % (metformin), 0.45 ± 0.05 % (moroxydine), 0.12 ± 0.01 % (neosaxitoxin), 0.14 ± 0.02 % (TTX), 0.11 ± 0.01 % (2'3'-cGAMP), 0.05 ± 0.01 % (teriparatide), 0.10 ± 0.04 % (insulin), and 0.31 ± 0.01 % (interleukin-2) release, which corresponds to a 7.1-fold (metformin, $P < 0.0001$), 4.1-fold (moroxydine, $P < 0.0001$), 8.6-fold (neosaxitoxin, $P < 0.0001$), 11.1-fold (TTX, $P < 0.0001$), 16.3-fold (2'3'-cGAMP, $P < 0.0001$), 5.5-fold (teriparatide, $P = 0.0012$), 5.5-fold (insulin, $P = 0.0046$) and 10.7-fold (interleukin-2, $P = 0.0001$) slower release compared to L-DSPC[0], respectively. P values are from unpaired two-tailed t test.

Rel-1h: release at 1 h.

Rel-7d: release at 7 days.



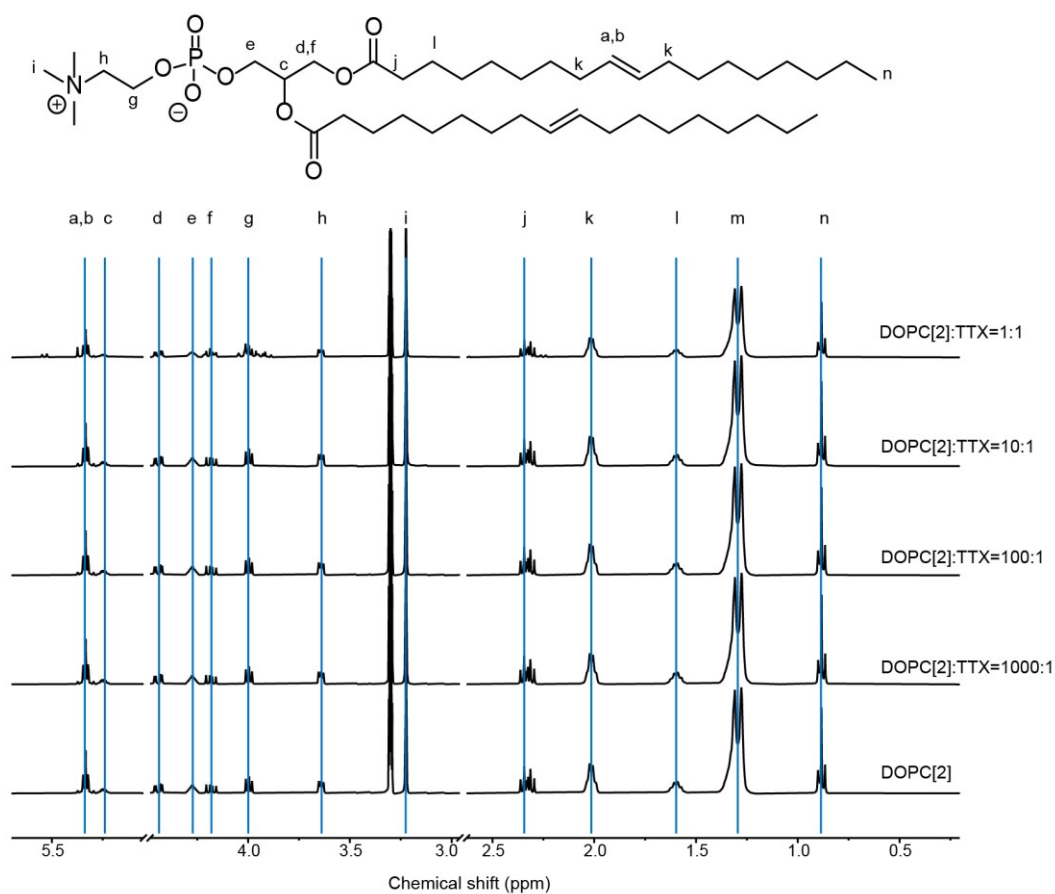
Supplementary Fig. 8. Effect of Log $P_{o/w}$ of hydrophilic drugs on release (Rel-1h and Rel-7d) from using L-DOPC[2] vs L-DSPC[0]. a, b, Rel-1h (a) and Rel-7d (b) (DSPC[0]/DOPC[2]) as a function of Log $P_{o/w}$.

EE: encapsulation efficiency.

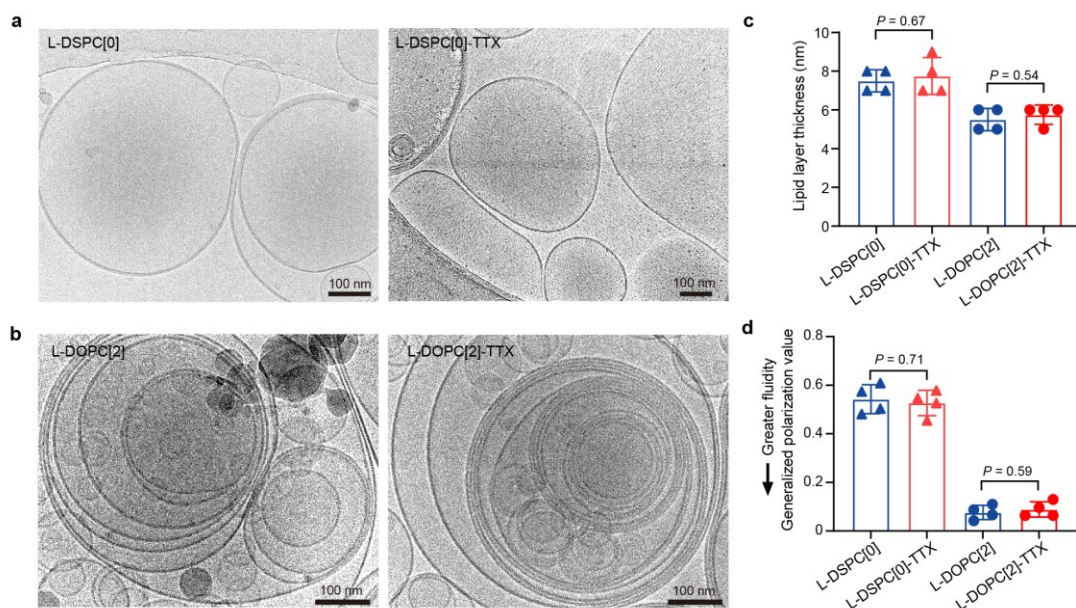
DL: drug loading.

Rel-1h: release at 1 h.

Rel-7d: release at 7days.

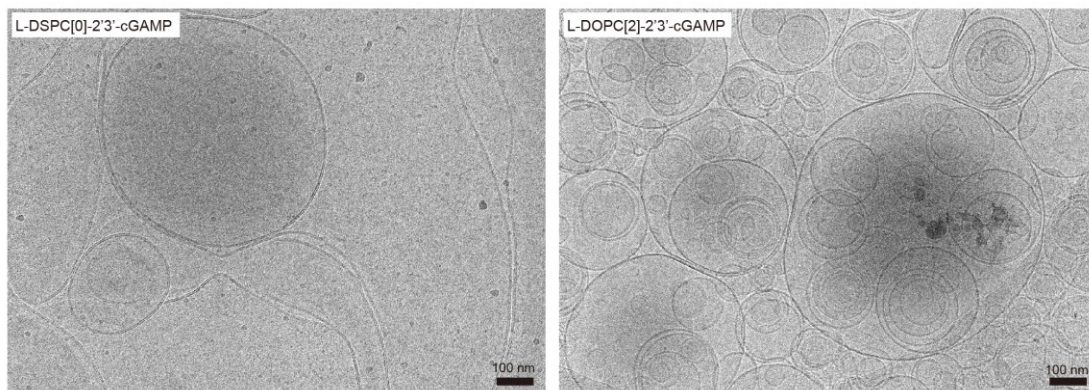


Supplementary Fig. 9. ¹H-NMR spectra of mixtures of DOPC[2] and TTX at varying molar ratios, in deuterated methanol. No chemical shifts were seen, suggesting no direct interactions between DOPC[2] and TTX.

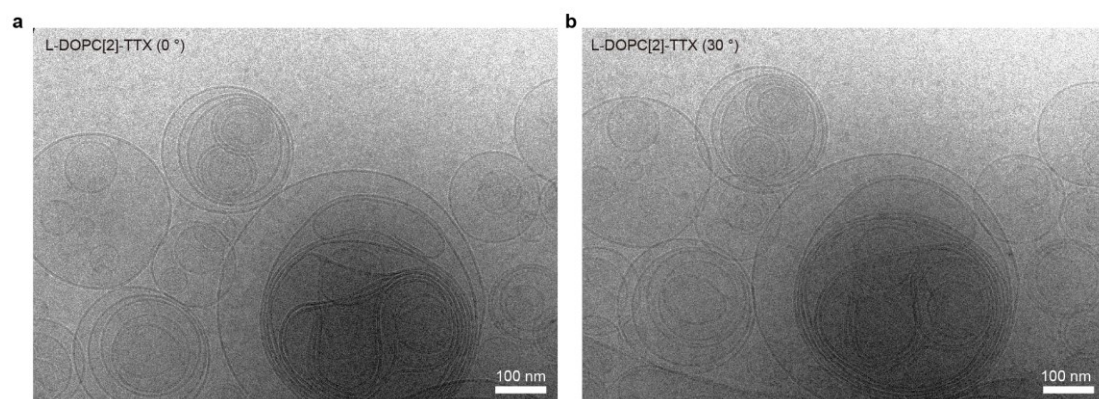


Supplementary Fig. 10. Effect of TTX on liposome properties. **a, b**, Representative (of 4 independent samples) cryogenic electron microscopy (cryo-EM) images of **(a)** L-DSPC[0] and **(b)** L-DOPC[2] with or without TTX. **c**, Lipid thickness of L-DSPC[0] and L-DOPC[2] with or without TTX. **d**, The Laurdan generalized polarization values of L-DSPC[0] and L-DOPC[2] with or without TTX. Data are means $\pm$ SD, $n = 4$ independent experiments. P values are from unpaired two-tailed t test.

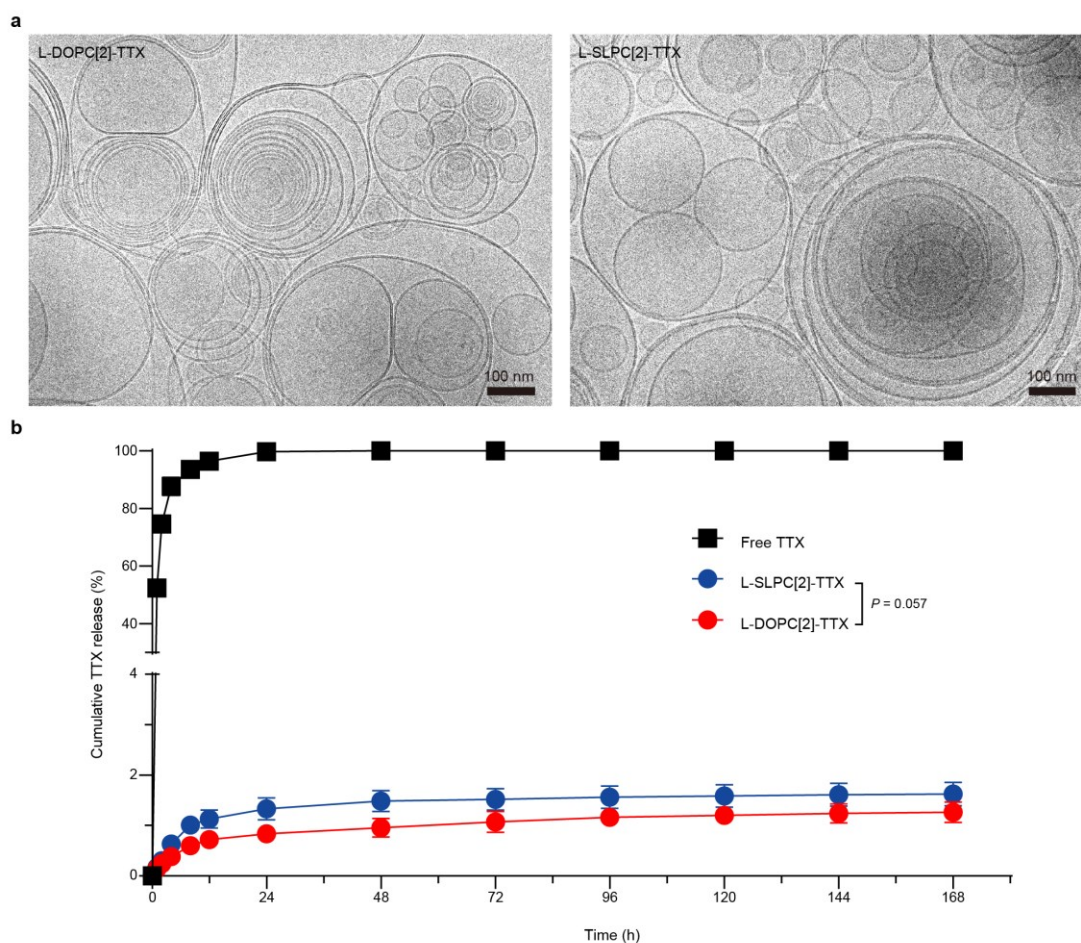
The presence of TTX did not change that L-DSPC[0] were unilamellar and L-DOPC[2] were ML/MV (Supplementary **Fig. 10a, b**). The thicknesses of the lipid bilayers were not changed by TTX: 7.5 nm for L-DSPC[0] and 7.8 nm for L-DSPC[0]-TTX ($P = 0.67$); 5.5 nm for L-DOPC[2] and 5.8 nm for L-DOPC[2]-TTX ($P = 0.54$) (Supplementary **Fig. 10c**). The lipid layer thickness was quantified from the cryo-EM images using ImageJ software. The membrane fluidity of the lipid bilayers was not changed by TTX (Supplementary **Fig. 10d**). The generalized polarization values (lower values = greater fluidity) were: 0.54 for L-DSPC[0] and 0.53 for L-DSPC[0]-TTX ($P = 0.71$); 0.08 for L-DOPC[2] and 0.09 for L-DOPC[2]-TTX ($P = 0.59$).



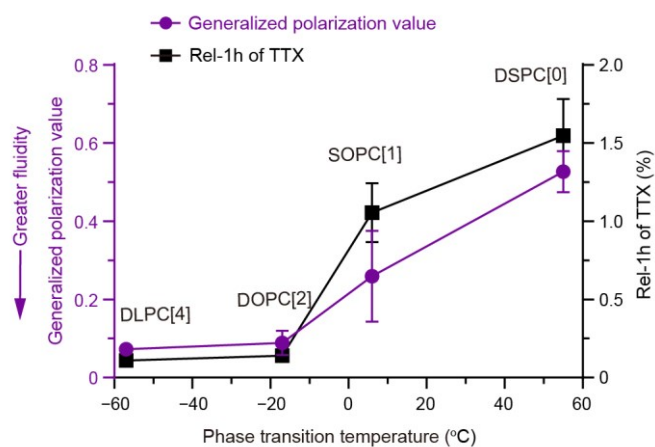
Supplementary Fig. 11. Representative (of 4 independent samples) cryo-EM images of L-DSPC[0]-2'3'-cGAMP and L-DOPC[2]-2'3'-cGAMP.



Supplementary Fig. 12. Representative (of 4 independent samples) cryo-EM images of liposomes (a) at 0 ° and (b) with a 30 ° tilt.



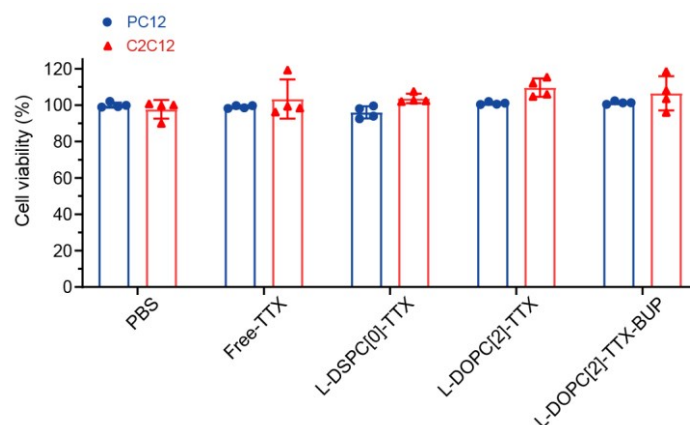
Supplementary Fig. 13. Structure and drug release of L-SLPC[2]-TTX and L-DOPC[2]-TTX. SLPC[2] and DOPC[2] have the same #DB (but with dienes in different locations) and charge. **a**, Representative (of 4 independent samples) cryo-EM images of L-DOPC[2]-TTX and L-SLPC[2]-TTX. **b**, Cumulative release of TTX from L-DOPC[2]-TTX and L-SLPC[2]-TTX. Data are means $\pm$ SD, $n = 4$ independent experiments. P value compares groups at 168 h with an unpaired two-tailed t test. #DB: number of double bonds in phospholipids.



Supplementary Fig. 14. Relation between T_m , membrane fluidity and TTX release (Rel-1h). Generalized polarization of Laurdan was used as the metric of membrane fluidity. Data are means $\pm$ SD, $n = 4$ independent experiments.

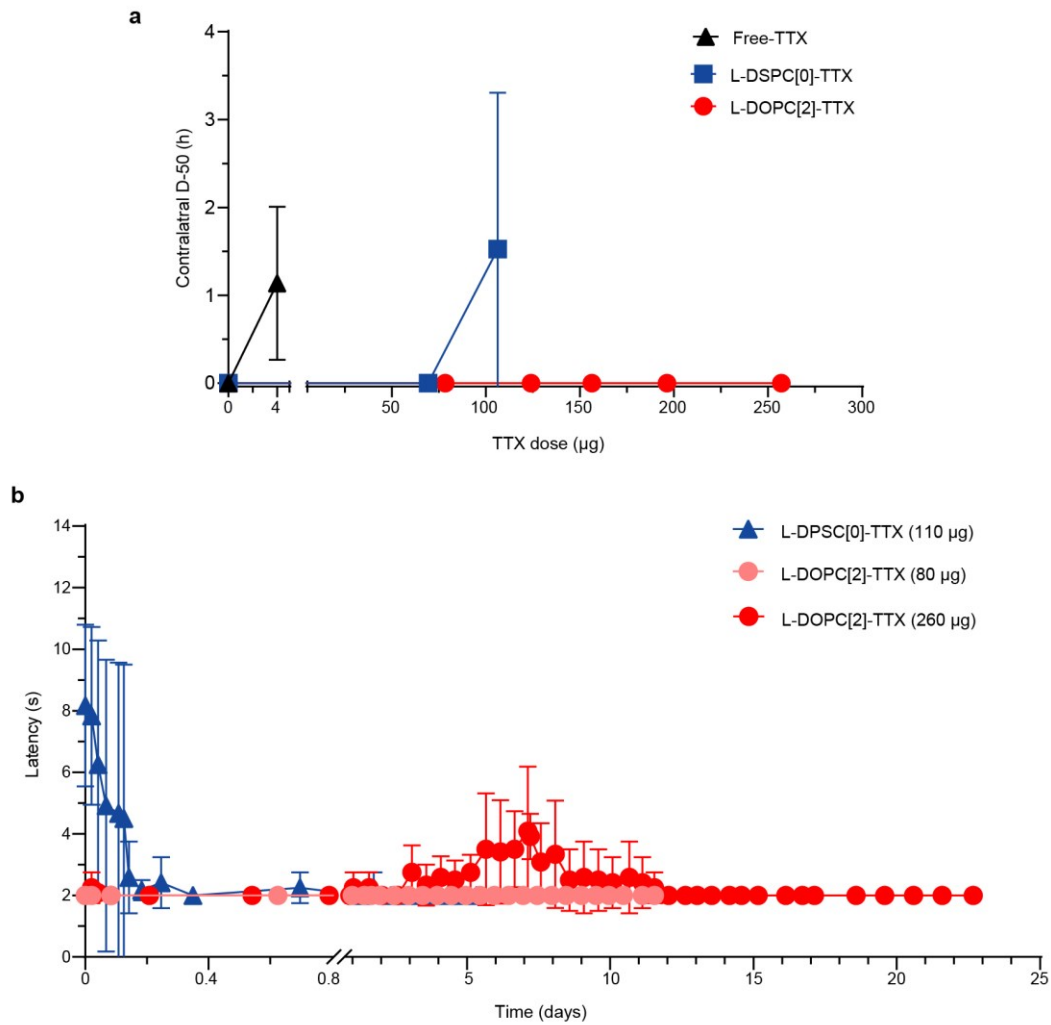
T_m : phase transition temperature.

Rel-1h: release at 1 h.



Supplementary Fig. 15. Cytotoxicity of formulations to PC12 and C2C12 cells. The TTX concentration was concentration was 2.6 mM; the BUP concentration was 1.2 mM; With liposomes, the lipid concentration was 21.3 mM. Cell viability is expressed as a percentage of that in cells treated with PBS. Data are means $\pm$ SD, $n = 4$ independent experiments.

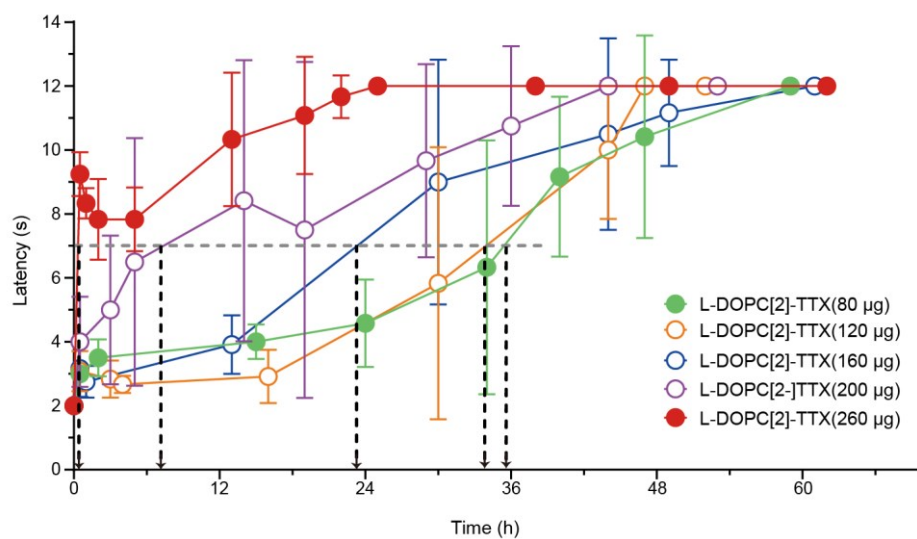
The *in vitro* cytotoxicity of TTX formulations was evaluated in myotube (differentiated C2C12 myoblast) and pheochromocytoma (PC12) cells, which are models for myotoxicity and neurotoxicity, respectively. After 48 h of incubation with the various liposome formulations tested *in vivo*, cell viabilities as assessed by MTS assays were $> 95\%$ in all groups.



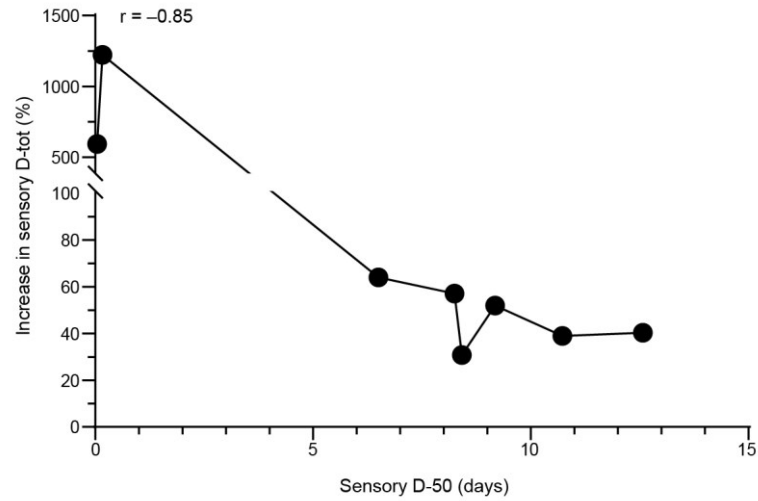
Supplementary Fig. 16. Contralateral block in selected TTX formulations. a, Sciatic nerve D-50 in the contralateral (uninjected) leg. **b,** Time courses of thermal latency in contralateral leg after injection of L-DSPC[0]-TTX(110 μg) or L-DOPC[2]-TTX (2 doses of TTX shown). Data are means $\pm$ SD, n = 4 rats per group.

Contralateral nerve blocks (i.e., latency $\geq$ 7s) from L-DSPC[0]-TTX(110 μg) were observed within 30 min of injection (the first time point tested), and lasted 1.5 ± 1.8 h. With L-DOPC[2]-TTX(260 μg), one rat (25%) showed a transient (< 1 h) contralateral block of 7 s at 7 days, two rats (50%) had lesser contralateral deficits (i.e., 4-6 s) after 5 days; in one rat this lasted for 1 day and in the other for 4 days.

D-50: the time for latency to return to half-maximal (i.e., a latency of 7 s).



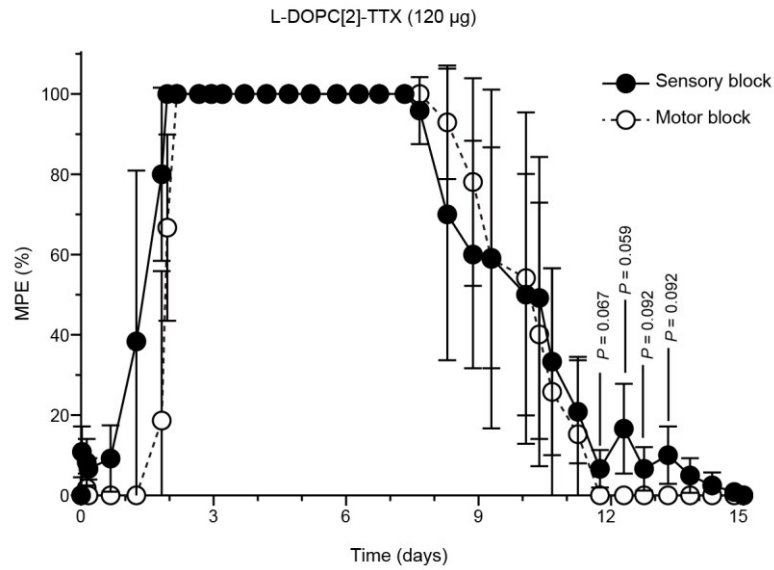
Supplementary Fig. 17. Onset of sciatic nerve block from TTX formulations. Effect of TTX dose on latency time course for L-DOPC[2]-TTX. Data are means $\pm$ SD, $n = 4$ rats per group. The grey dashed line indicates a latency of 7s; the dashed black arrows indicate the time to reach that latency (i.e., time to onset). The time to onset at various TTX loadings were: 39 ± 10 h (80 μ g); 35 ± 9 h (120 μ g); 30 ± 11 h (160 μ g); 17 ± 15 h (200 μ g); 0 h (260 μ g).



Supplementary Fig. 18. Sensory D-tot. The increase ratio of sensory D-tot to D-50 as a function of sensory D-50.

D-50: the time for latency to return to half-maximal (i.e., a latency of 7 s).

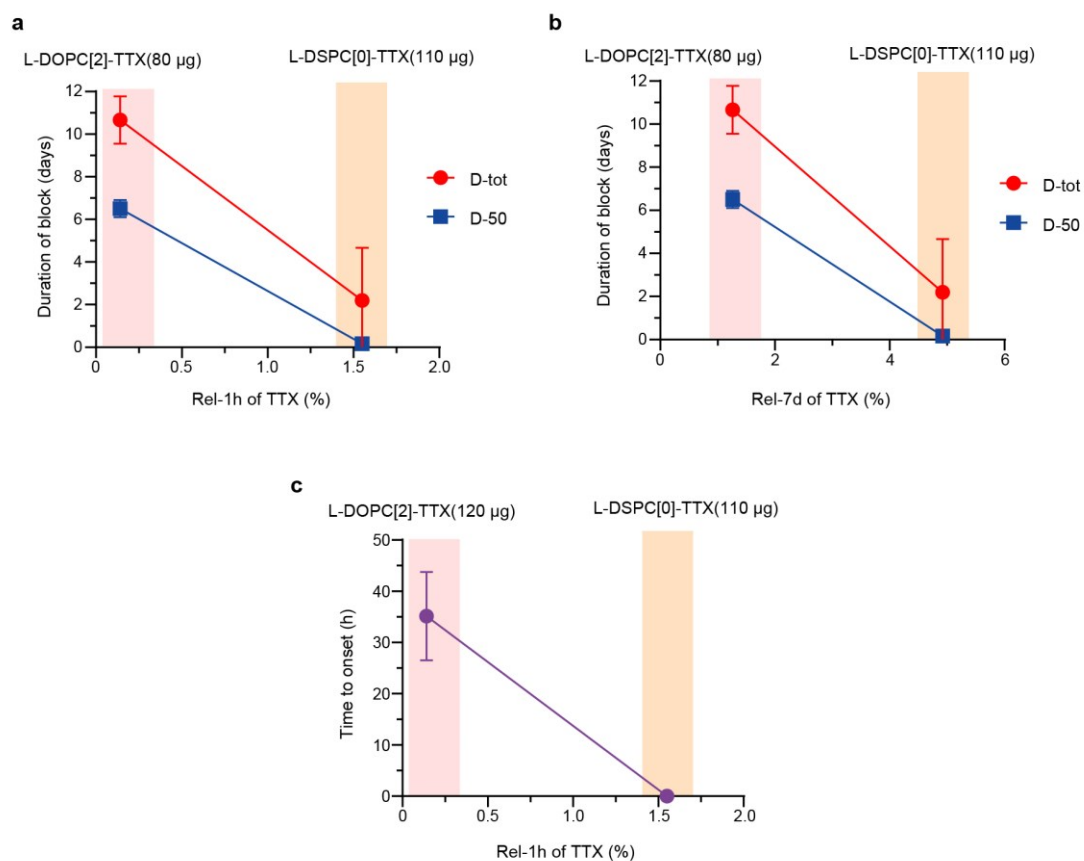
D-tot: the time for latency to return to baseline latency (i.e., a latency of 2 s).



Supplementary Fig. 19. Sensory D-tot vs motor D-tot. Time course of sensory and motor block from L-DOPC[2]-TTX(120 μ g). The anesthetic effects (latency and weight bearing) are presented as the percent maximum possible effect, %MPE. Data are means $\pm$ SD, $n = 4$ rats per group. P values compare groups with paired two-tailed t test.

Sensory D-tot: the time for latency to return to baseline latency (i.e., a latency of 2 s).

Motor D-tot: the time for motor strength to return to maximum strength before injection.



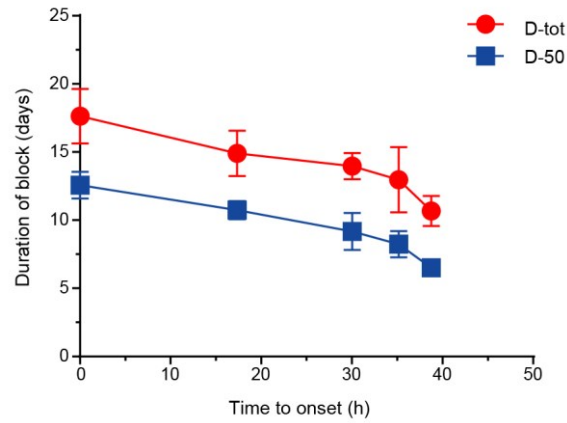
Supplementary Fig. 20. Relation of *in vivo* results (duration of block [D-50 and D-tot] and time to onset) to *in vitro* release rates of TTX. a, b, D-50 and D-tot vs. (a) Rel-1h and (b) Rel-7d. c, Time to onset vs. Rel-1h of TTX. Data are means $\pm$ SD, n = 4 independent experiments.

Rel-1h: release at 1 h.

Rel-7d: release at 7 days.

D-50: the time for latency to return to half-maximal (i.e., a latency of 7 s).

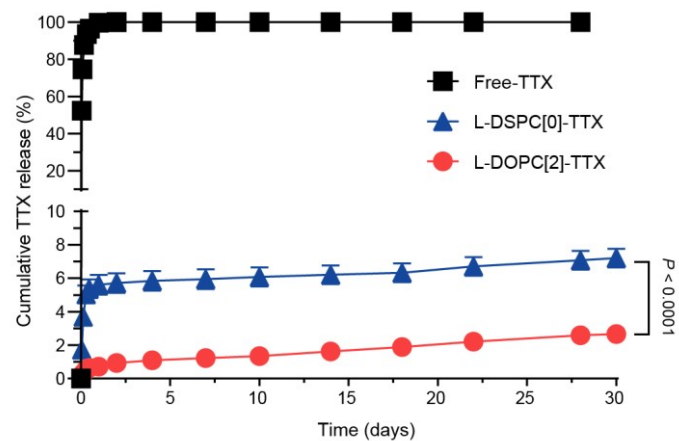
D-tot: the time for latency to return to baseline latency (i.e., a latency of 2 s).



Supplementary Fig. 21. Relation of time to onset to duration of block with L-DOPC[2]-TTX. The TTX doses are indicated in Fig. 4f. Data are means $\pm$ SD, $n = 4$ independent experiments.

D-50: the time for latency to return to half-maximal (i.e., a latency of 7 s).

D-tot: the time for latency to return to baseline latency (i.e., a latency of 2 s).



Supplementary Fig. 22. Cumulative release (%) of TTX from L-DSPC[0] and L-DOPC[2]. Data are means $\pm$ SD, $n = 4$ independent experiments. P value compares groups at 30 days with an unpaired two-tailed t test.

Supplementary Reference

1. Guillén, M.D. & Ruiz, A. Monitoring the oxidation of unsaturated oils and formation of oxygenated aldehydes by proton NMR. *Eur. J. Lipid Sci. Technol.* **107**, 36-47 (2005).