supporting information

A Fast-Acting Brain-Targeted Nano-Delivery System with Ultra-Simple Structure for Brain Emergency Poisoning Rescue

Yao Li*, Zinan Zhang*, Jingyi Huang, Huanchun Xing, Lin Wang, Lijuan Huang, Xin Sui, Yuan Luo, Liao Shen†, Yongan Wang†, and Jun Yang†

Experimental Section

Preparation of the BTNDS.

Six types of thermosensitive liposomes, including DPPC, SOPC, MPPC, POPS, DPPS, and DMPS, were prepared using the film-ultrasonic method. Briefly, the thermosensitive liposomes were first dissolved in chloroform, followed by the evaporation of the organic solvent using a rotary evaporator at room temperature to form a membrane. The antidote or fluorescent dye solutions were mixed with the membrane and sonicated to form a preliminary liposome suspension. Then, the preliminary liposome suspension was extruded through a 0.1 μm polycarbonate membrane using a syringe extruder 10 times to obtain a narrow size distribution (Fig. 1c, left). For mass production, the preliminary liposome suspension was extruded using a professional high-pressure extruder. (Fig. 1c, right). The final products were stored at 4 °C.

Determination of the Drug Loading Ratio (DLR) and the Percentage of Drug Entrapment (PDE).

The DLR and PED were measured using the ultrafiltration method28. Briefly, 0.5 ml of the liposome suspension was separated by centrifugal filter (Merck Millipore, 15 min, 10000 g, 4 °C) . The volume and concentration of the filtrate in the lower tubes of centrifugal filter were then measured. The DLR and PED were calculated using the following equations:

DLR = (total amount of drug-free drug) /total amount of product ×100%. [1]

PDE = (total amount of drug-free drug)/ total amount of drug × 100%. [2]

Determination of the drug release in vitro and in vivo.

Four milliliters of POPS-based nanoparticles loaded with HI-6 were placed in a dialysis bag1, which was suspended in 900 ml physiological saline solution at 37 °C. After a predetermined period, 2 ml of the solution was taken from the system for High-performance liquid chromatography, and 2 ml of fresh solvent was added. To further investigate the release behavior, BTNDS composed of different thermosensitive liposomes (DPPC, SOPC, MPPC, DPPS) were prepared and tested as mentioned above.

Animals.

All experiments were performed in accordance with the Regulations of the Experimental Animal Administration, issued by the State Committee of Science and Technology of the People's Republic of China (November 14, 1988), and with the Guidelines for Care and Use of Laboratory Animals of the Beijing Institute of Pharmacology and Toxicology and the experiments were approved by the Animal Ethics Committee of the Beijing Institute of Pharmacology and Toxicology. Kunming mice (4 weeks old) were purchased from the Animal Center of the Academy of Military Science. Transgenic Thy-1-YFP mice were purchased from the Jackson Laboratory (Stock No: 003709). The rodents had free access to sterilized food and distilled water and were maintained in stainless steel cages filled with hardwood chips in an air-conditioned room under 12:12 h light/dark cycles.

Establishing the NA poisoning mouse model.

Fifty mice were randomly allocated to five groups and subcutaneously injected with soman in the neck at the doses of 30, 60, 100, 120, and 200 μg/kg (injection volume: 10 μL/g). Toxicity symptoms were recorded for 24 h.

Determination of the ARR.

Kunming mice were randomly divided into 7 groups (n = 10/group): untreated non-poisoned controls (group 1), untreated soman-poisoned (120 μg/kg soman; group 2), soman-poisoned and treated with aqueous HI-6 solution (2.2 mg/ml solution at a dose of 10 μL/g; group 3), soman-poisoned and treated with the DPPC-based BTNDS (8.0 mg/ml with 2.2 mg/ml HI-6 at a dose of 10 μL/g; group 4), soman-poisoned and treated with the MPPC-based BTNDS (8.0 mg/ml with 2.2 mg/ml HI-6 at a dose of 10 μL/g; group 5), soman-poisoned and treated with the SOPC-based BTNDS (8.0 mg/ml with 2.2 mg/ml HI-6 at a dose of 10 μL/g; group 6), and soman-poisoned and treated with the POPS-LPs (8.0 mg/ml with 2.2 mg/ml HI-6 at a dose of 10 μL/g; group 7). The antidote was injected via the tail vein immediately after soman poisoning. Blood and brain samples were collected 10 minutes later. The brain tissues were homogenized followed by centrifugation to obtain the supernatants for subsequent analyses. Twenty microliters of blood or tissue supernatant diluted 50-fold was added per well of an EIA/RIA plate (Costar 9018; Sigma-Aldrich, St. Louis, MO, USA). Adrenocorticotrophic hormone (30 μL of a 1% solution) and PBS (30 μL of a 0.1 M solution) were added to three wells each. After incubation for 30 min at 37 °C, 5,5-dithiobis-2-nitrobenzoic acid (200 μL of a 1% solution) was added to each well and the optical density (OD) was measured at a wavelength of 415 nm. The ARR was calculated using the following equation:

ARR (%) = (OD415 nm [Group 3-7] − OD415 nm [Group 2])/(OD415 nm [Group 1] − OD415 nm [Group 2]). [3]

The determination of the ARR under moderate poisoning conditions was performed in a similar fashion; the only difference was the soman poisoning dose used - 100 μg/kg. Additionally, the ARR was also determined as explained above in the context of DMPS-LPs and POPS-LPs.

Evaluation of the cytotoxicity in vitro.

BMECs were incubated with HI-6 or POPS-LPs at concentrations of 0 (control), 0.001, 0.01, 0.1, 1, 10, and 100 μg/ml for 48 h. After washing with PBS, the cells were incubated with CCK-8 (10 μL) for 4 h and then the absorbance at 450 nm was measured.

Evaluation of the survival rate and time of poisoned animals.

Kunming mice were poisoned with different concentrations of soman - 120, 140, 160, 180, 200, 240, 260, 280, and 300 μg/kg. Thereafter, animals were either kept untreated (group 1), or treated with aqueous HI-6 solution (2.2 mg/ml solution at a dose of 10 μL/g, group 2), or the POPS-BTNDSLPs (8.0 mg/ml, 2.2 mg/ml HI-6 at a dose of 10 μL/g, group 3). Ten mice were administrated per concentration. The survival rate of mice was determined per poisoning concentration based on the animals alive, 24 h after treatment. Additionally, the survival time was also recorded in the context of animals poisoned with soman at 340 μg/kg.

Histopathological analysis of brain sections.

Kunming mice were divided into four – the three mentioned above, poisoned at 300 μg/kg, plus a non-poisoned control group. After treatment for 24 h, the brains were collected and fixed in glutaraldehyde (3%) for 48 h. After dehydration and paraffin embedding, the tissues were cut into 4 μm sections and stained with H&E. The sections were visualized using light microscopy.

Determination of the cell uptake of liposomes in vitro.

FLU was used as a fluorescent reporter. Briefly, BMECs were incubated with FLU and POPS-FLU at a concentration of 5 μg/ml. At predetermined time points (0.5, 1, and 4 h), cells were washed with PBS, stained with DAPI, and imaged via laser scanning confocal micorscope (LSCM, excitation wavelength: 405 nm for DAPI and 520 nm for FLU). HCl was also used for quantitative evaluation. With this respect, BMECs were incubated with FLU and POPS-FLU at concentrations of 0.3125, 0.625, 1.25, 2.5, 3.75, 5, 6.25, 7.5, and 8.75 μg/ml for 1 h, followed by fixation, and DAPI staining; in parallel, BMECs were incubated with FLU and POPS-FLU at 5 μg/ml for 0.5, 1, 2, and 4 h, followed by fixation, and DAPI staining. All BMECs were then imaged via this, and the quantitative analysis was performed using the system software.

The quantitative analysis of cellular uptake about the PLA2 impact was also performed. BMECs were incubated with the FLU, POPS-FLU, FLU plus PLA2 (PLA2 0.1 mg/ml) and POPS-FLU plus PLA2 (PLA2 0.1 mg/ml), either at FLU concentrations of 50, 62.5, 75, 87.5, 100 μg/ml for 1 h, or at a fixed FLU concentration (100 μg/ml) for 0.5, 1, 2, and 4 h, followed by fixation and imaging using HCI. The influence of AChE on the BBB permeability was also determined using the same experimental layout.

Determination of the internalization route of the nanoparticles.

BMECs were incubated with the fluorescent dye Cy3-α-Bungarotoxin, dissolved in Live cell imaging solution (Thermo Fisher, A1429DJ) at a concentration of 0.1 mg/ml (marker of the acetylcholine receptor-mediated internalization), A488-Transferrin (0.5 mg/ml; marker of the transferrin receptor-mediated internalization), or FITC-dextran (0.005 mg/ml; internalized via the endocytosis pathway) for 3 h. After washing with PBS three times, the cells were incubated with POPS-FLU for 3 h and then stained with DAPI. The fluorescently labeled cells were then imaged via LSCM. (excitation wavelength: 405 nm for DAPI, 520 nm for FLU, and 550 nm for Cy3), and co-localization of the different signals was determined.

Evaluation of the targeting of the central nervous system in vivo.

Kunming mice were divided into two groups and treated with either Cy3 or POPS-Cy3 (30 μg/kg). At predetermined time points (10 min, and 0.5, 1, 4, 12, and 24 h), the mice were anesthetized and perfused to eliminate the interference of peripheral residual fluorescence. After fixing in glutaraldehyde (3%) for 24 h, the brain was cut into 4 μm frozen sections and stained with DAPI. The sections were visualized using LSCM (excitation wavelength: 405 nm for DAPI and 550 nm for Cy3). Additionally, a Live fluorescence imaging system was also used; mice were administered POPS-Dio (1000 μg/kg) and imaged at predetermined periods (0.5, 1, 4, 12, and 24 h), at an excitation wavelength of 750 nm. The main organs, including the brain, heart, liver, kidney, spleen, and lungs, were also dissected and imaged individually under the same conditions.

Quantification of the levels of PLA2.

Mice were subcutaneously injected in the neck with soman at a dosage of 120 μg/kg. At predetermined time points (10 min, 1 h, and 1, 3, 5, and 7 d), blood was collected and centrifuged (15 min, 2000 g, 4 °C) to obtain the serum. Then, the content of PLA2 in the serum was measured using a commercially PLA2 ELISA kit (MB-3210B, Meibiao Biology Co., Ltd), as per the manufacturer's instructions.

Transwell in vitro experiments.

Highly active BMECs were isolated from 2-week-old Sprague Dawley rats via two-step enzyme digestion, followed by density gradient centrifugation, and differential adhesion. The primary cells were then plated on the membrane of the Transwell system at a seeding density of 2 × 105 cells/ml, and the medium was changed every 2–3 days. After a period of time, a barrier layer formed.

Additionally, the permeability to drugs was also determined using the transwell system. Briefly, different cell layers in the transwell plates were treated with FLU (FLU 0.1 mg/ml, 100 μL), POPS--FLU (FLU 0.1 mg/ml, 100 μL), FLU plus PLA2 (FLU 0.1 mg/ml, 100 μL, PLA2 0.1 mg/ml, 10 μL), and POPS-FLU plus PLA2 (FLU 0.1 mg/ml, 100 μL, PLA2 0.1 mg/ml, 10 μL). Samples were collected from the lower chamber 10, 30, and 60 min after treatment, and fluorescence was measured at an excitation wavelength of 520 nm. The permeability coefficient was calculated based on previous reports2

Determination of the impact of PLA2 on brain targeting in vivo.

Kunming mice were divided into two series: one series was administered soman (120 μg/kg) 7 days earlier to increase the endogenous PLA2 content, and injected with Cy3 (0.1 mg/ml) or POPS-LPs-Cy3 (0.1 mg/ml). The un-poisoned series was divided into five groups treated with Cy3 (0.1 mg/ml), POPS-Cy3 (0.1 mg/ml), Cy3 plus PLA2 (Cy3 0.1 mg/ml, PLA2 0.1 mg/ml,), POPS-Cy3 plus PLA2 (Cy3 0.1 mg/ml, PLA2 0.1 mg/ml), and vehicle. One hour after treatment, the mice were anesthetized and perfused. Frozen brain sections were then obtained, stained with DAPI, and visualized using LSCM (excitation wavelength: 405 nm for DAPI and 550 nm for Cy3).

In a different experimental setting, EB was loaded into POPS nanoparticles (POPS-EB) for vascular permeability evaluation. Kunming mice were also divided into three groups; two groups were poisoned as mentioned above. The poisoned mice were administered EB (0.5% v/v), POPS-EB (POPS 0.1 mg/ml, EB 0.5% v/v), EB plus PLA2 (EB 0.5%, PLA2 0.1 mg/ml), and POPS-Cy3 plus PLA2 (EB 0.5% v/v, PLA2 0.1 mg/ml).

Some poisoned animals were intraperitoneally injected with the PLA2 inhibitor Varespladib (1 mg/ml, 1 ml) and treated 1 h after with EB and POPS-EB as mentioned earlier. The unpoisoned mice were administered the same antiodotes. Finally, their brains were processed and imaged as described above.
Last but not least, A Live fluorescence imaging reporter was also used. The fluorescence-labeled PLA2 (PLA2-Cy7) for in vivo imaging was synthesized as follows: Cy7-NHS (1 mg) was reacted with PLA2 (10 mg) in 3 ml PBS at 4 °C without light for 4 h. The unreacted Cy7-NHS was removed using a Millipore ultrafiltration tube (3 K). In parallel, POPS-LPs loaded with Cy7 (POPS-Cy7) were also prepared using a previously described method. Kunming mice were injected in the tail vein with Cy7 (10 μg/ml, 5 μL/g), Cy7 plus PLA2 (Cy7 10 μg/ml, 5 μL/g, PLA2 0.1 mg/ml), POPS-Cy7 (Cy7 10 μg/ml, 5 μL/g), POPS-Cy7 plus PLA2 (Cy7 10 μg/ml, 5 μL/g, PLA2 0.1 mg/ml), or PLA2-Cy7 (0.1 mg/ml), and subjected to in vivo live imaging at predetermined time points (0.5, 1, 4, 12, and 24 h) with excitation wavelengths of 740 nm. Different organs, including the brain, heart, liver, kidney, spleen, and lungs were also dissected imaged individually, as described above.

Evaluation of the brain uptake of POPS nanoparticles.

Kunming mice were divided into two series. One series was poisoned with soman (120 μg/kg) and treated 7 days later with Cy7 (10 μg/ml), POPS-Cy7 (Cy7 10 μg/ml), or POPS-Cy7 (Cy7 10 μg/ml) plus Varespladib (intraperitoneal injection 1 h before treatment, 1 mg/ml, 1 ml). The unpoisoned series was injected with Cy7 (10 μg/ml) and POPS-Cy7 (10 μg/ml). One hour after treatment, the mice were anesthetized perfused. Their brains were collected, weighed, and homogenized in 2 ml PBS followed by centrifugation to obtain the supernatants. The fluorescence of the supernatants was then measured at an excitation wavelength of 740 nm.

Evaluation of the effectiveness of the improved nano-antidote.

Kunming mice were poisoned with different soman concentrations and divided into four series: soman-poisoned (series 1), soman-poisoned and treated with aqueous HI-6 solution plus PLA2 (HI-6 2.2 mg/ml solution, PLA2 0.1 mg/ml at a dose of 10 μL/g, series 2), soman-poisoned and treated with the POPS-LPs plus PLA2 (HI-6 2.2 mg/ml solution, PLA2 0.1 mg/ml at a dose of 10 μL/g, series 3), and soman treated with PLA2 (0.1 mg/ml, series 4). The survival rate was determined, as described previously. The survival time was also determined, as previously described in animals poisoned with 340, 440, and 640 μg/kg of soman and treated with the series of antidotes as former. Three different concentrations of HI-6 were used (n=10 per group): 1.1, 2.2, 4.4 mg/ml. Additionally, the ARR was also determined as above in the brain and blood of soman-poisoned Kunming mice treated with aqueous HI-6 (2.2 mg/ml), the POPS-LPs (2.2 mg/ml, group 4), and the POPS-LPs plusPLA2 (HI-6 2.2 mg/ml, PLA2 0.1 mg/ml).

Use of the Thy-1-YFP reporter mouse model to observe brain neurotoxicity.

Thy-1-YFP mice were poisoned with 60 μg/kg soman, and treated with HI-6 aqueous (2.2 mg/ml), the POPS-LPs (2.2 mg/ml), HI-6 aqueous plusPLA2 (HI-6 2.2 mg/ml, PLA2 0.1 mg/ml), the POPS-LPs plus PLA2 (HI-6 2.2 mg/ml, PLA2 0.1 mg/ml), or saline. Twenty-four hours later, the brains were collected, fixed, stained with DAPI, and imaged via LSCM (excitation wavelength: 405 nm for DAPI and 520 nm for GFP).

Statistical analysis.

Statistical differences were assessed using the one-way analysis of the variance (ANOVA) followed by Newman–Keuls or Tukey's honestly significant difference post-hoc tests. The criterion for statistical significance was set at $P < 0.05$.

1 Y. Zhang, J. He, L. Shen, T. Wang, J. Yang, Y. Li, Y. Wang and D. Quan, Journal of Controlled Release, 2021, 329, 1117-1128.
2 N. Perrière, P. Demeuse, E. Garcia, A. Regina, M. Debray, J. P. Andreux, P. Couvreur, J. M. Scherrmann, J. Temsamani and P. O. Couraud, Journal of Neurochemistry, 2010, 93, 279-289.

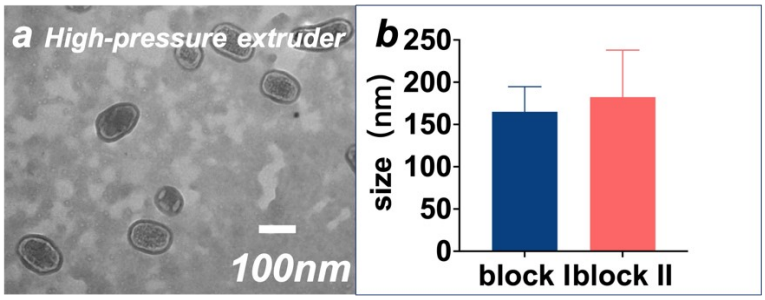

Fig. S1. a, TEM images of POPS-LPs prepared by High-pressure extruder. b, The size of different POPS-LPS prepared by High-pressure extruder for two batches, as characterized by dynamic light scattering. N=3.

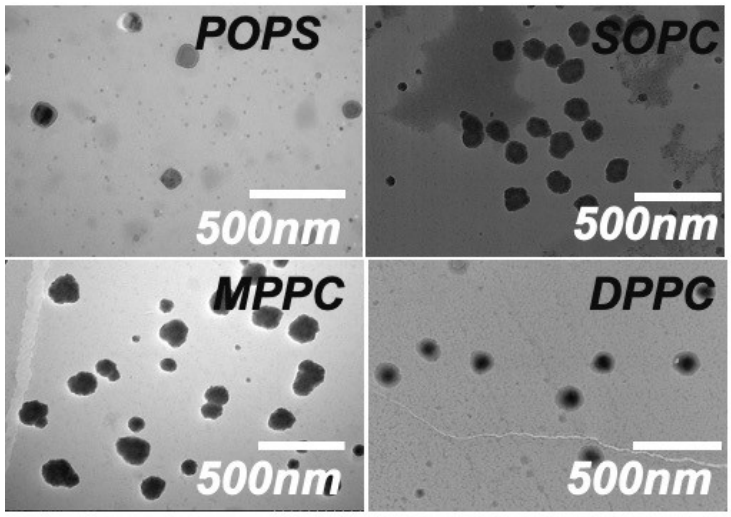

Figure S2 TEM images of POPS-, SOPC-, MPPC- and DPPC-based nanoparticles (from up to down). Scale bar, 500 nm.

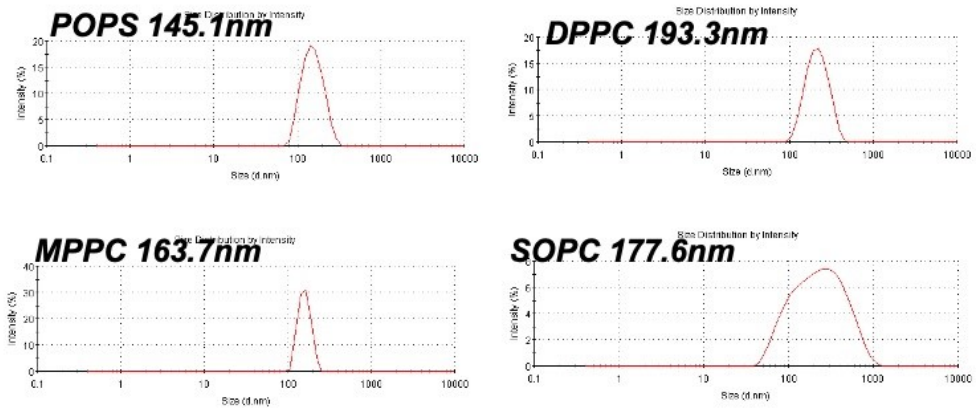

Figure S3 The size of different BTNDS, as characterized by dynamic light scattering.

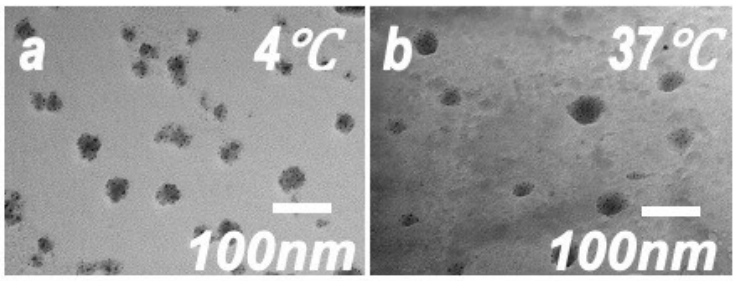

Figure S4 TEM images of POPS-LPs under 4℃ storage and 37 ℃ incubation period over 7 days.

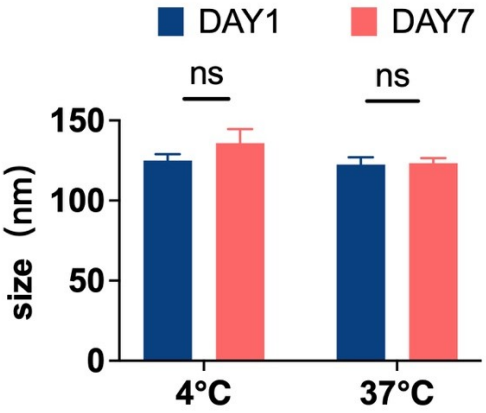

Figure S5 The dynamic light scattering size of POPS-LPs under 4℃ storage and 37 ℃ incubation period over 7 days.

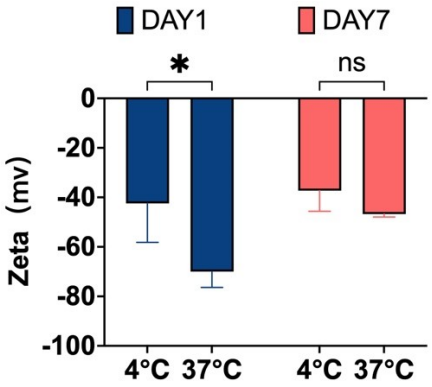

Figure S6 The zeta potential of POPS-LPs under 4℃ storage and 37 ℃ incubation period over 7 days.

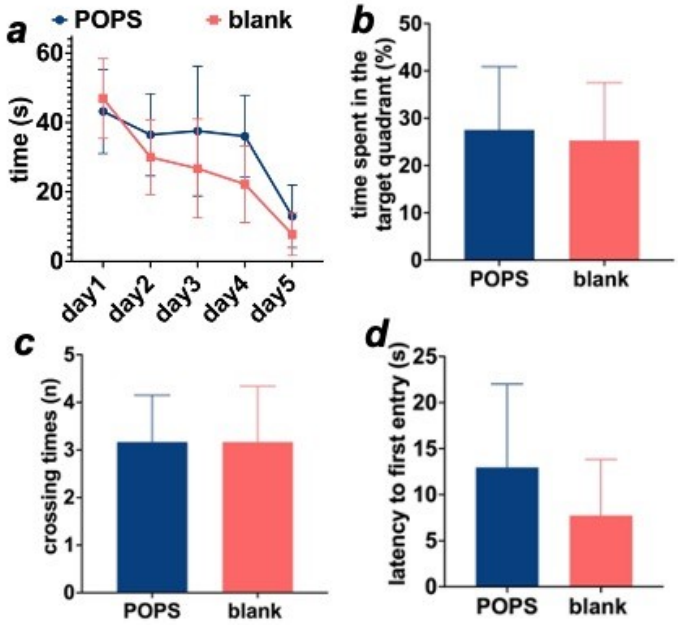

Figure S7 Effect of POPS on the cognitive performance of mice in the Morris water maze. Mice were treated with POPS for 2 days. (A) Escape latency during the training days. (B) Percentage of time spent in the target quadrant on day 5. (C) Number of platform crossings on day 5. (D) Escape latency.

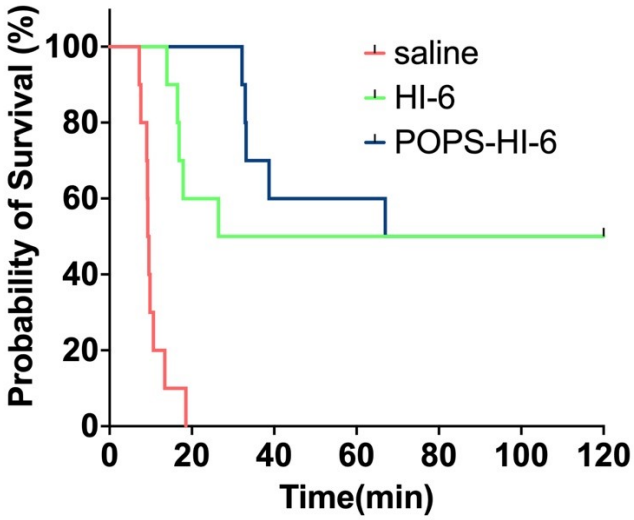

Figure S8 The survival time of mice poisoned with 220 μg/kg soman and then not treated (red), or treated with HI-6 (green) or the POPS-LPs (blue). (N=10 per group).

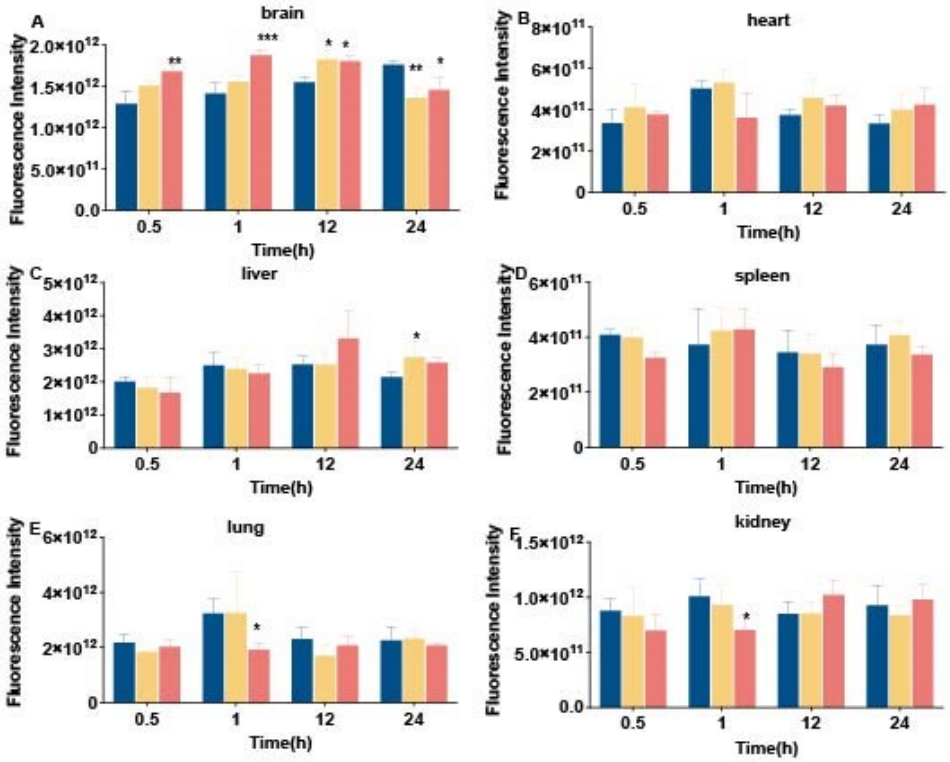

Figure S9 the fluorescence intensity of organs from mice treated with cy7 (blue), cy7-marked POPS-LPs (yellow) and cy7-marked POPS-LPs mixing with PLA2 (red) during 24 h.