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Supplementary Materials for

A new painkiller nanomedicine to bypass the blood-brain barrier and the use of morphine

Jiao Feng, Sinda Lepetre-Mouelhi, Anne Gautier, Simona Mura, Catherine Cailleau, François Coudore, Michel Hamon, Patrick Couvreur*

*Corresponding author. Email: patrick.couvreur@u-psud.fr

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Supplementary Text
IR, NMR and MS characterization of bioconjugates

IR spectra were obtained from solids or neat liquids with a PerkinElmer UATR Two spectrometer. Only significant absorptions are listed. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 400 spectrometer (400 and 100 MHz for ^{1}H and ^{13}C, respectively). Recognition of methyl, methylene, methine, and quaternary carbon nuclei in ^{13}C NMR spectra rests on the J-modulated spin-echo sequence. Mass spectra were recorded on a Bruker Esquire-LC. High resolution Mass spectra (HR-MS) were achieved with an LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific) operating in positive and negative electrospray ionization.

IR, NMR and MS characterization of LENK-SQ-Diox: IR (neat, cm^{-1}): ν 3289, 2958, 2916, 2849, 1763, 1646, 1537, 1515, 1447, 1381, 1259, 1116, 1020, 982, 870, 802, 729, 700, 549, 493. ^{1}H NMR (400 MHz, MeOD) δ: 7.31-7.23 (m, 4H, $2H_{Ar\text{-}ortho}$ Phe, $2H_{Ar\text{-}meta}$ Phe), 7.18 (m, 1H, $H_{Ar\text{-}para}$ Phe), 7.04 (d, 2H, $H_{Ar\text{-}ortho}$ Tyr, *J* = 8.4 Hz), 6.71 (d, 2H, $H_{Ar\text{-}meta}$ Tyr, *J* = 8.4 Hz), 5.77 (d, 1H, O*C*H_2O, *J* = 5.6 Hz), 5.71 (d, 1H, O*C*H_2O, *J* = 5.6 Hz), 5.19–5.04 (m, 5H, *H*C=C(CH_3)), 4.65 (dd, 1H, CH Phe, *J* = 4.9 Hz, *J* = 9.6 Hz), 4.44 (m, 1H, CH Leu), 4.00-3.60 (m, 4H, 2 CH_2 Gly), 3.54 (dd, 1H, CH Tyr, *J* = 6.5 Hz, *J* = 7.6 Hz), 3.16 (dd, 1H, C*Ha*Hb Phe, *J* = 4.9 Hz, *J* = 14.0 Hz), 3.10-2.87 (m, 2H, CHa*Hb* Phe, C*Ha*Hb Tyr), 2.80 (dd, 1H, CHa*Hb* Tyr, *J* = 7.6 Hz, *J* = 13.9 Hz), 2.44 (m, 2H, CH_2-CH_2-CO SQ), 2.26 (m, 2H, CH_2-CH_2-CO SQ), 2.14-1.90 (m, 16H, 8 CH_2 SQ), 1.75-1.48 (m, 21H, CH_2 Leu, C*H*(CH_3)$_2$ Leu, 6 CH_3 SQ), 0.94 (d, 3H, CH_3 Leu, *J* = 6.2 Hz), 0.90 (d, 3H, CH_3 Leu, *J* = 6.2 Hz). ^{13}C NMR (75 MHz, MeOD) δ: 178.0 (CONH), 173.7 (CONH), 173.0 (CONH), 172.4 (CONH), 172.0 (CONH), 171.3 (CONH), 157.6 ($C_{Ar\text{-}para}$ Tyr), 138.3 (C_{Ar} Phe), 136.0 (HC=*C*(CH_3)), 135.8 (2 HC=*C*(CH_3)), 134.1 (HC=*C*(CH_3)), 132.0 (HC=*C*(CH_3)), 131.5 (2 $CH_{Ar\text{-}ortho}$ Tyr), 130.4 (2 $CH_{Ar\text{-}ortho}$ Phe), 129.5 (2 $CH_{Ar\text{-}meta}$ Phe, C_{Ar} Tyr), 127.8 ($CH_{Ar\text{-}para}$ Phe), 126.5 (H*C*=C(CH_3)), 125.7 (H*C*=C(CH_3)), 125.5 (2 H*C*=C(CH_3)), 125.4 (H*C*=C(CH_3)), 116.5 (2 $CH_{Ar\text{-}meta}$ Tyr), 80.9 (O-CH_2-O), 62.6 (CH Tyr), 55.8 (CH Phe), 52.2 (CH Leu), 43.8 (CH_2 Gly), 43.6 (CH_2 Gly), 41.0 (*C*H_2-CH(CH_3)$_2$ Leu), 40.8 (CH_2 SQ), 40.7 (2 CH_2 SQ, CH_2 Tyr), 38.7 (CH_2 Phe), 35.3 (*C*H_2-CH_2-CO), 33.8 (CH_2-*C*H_2-CO), 30.7 (CH_2 SQ), 30.4 (CH_2 SQ), 29.2 (CH_2 SQ), 27.8 (CH_2 SQ), 27.5 (CH_2 SQ), 25.9 (*C*H(CH_3)$_2$ Leu), 23.4 (CH_3 Leu), 21.9 (CH_3 Leu), 17.8 (CH_3 SQ), 16.7 (CH_3 SQ), 16.2 (CH_3 SQ), 16.1 (CH_3 SQ), 16.0 (CH_3 SQ), 14.5 (CH_3 SQ). HRMS (+ESI): *m/z* 968.6064 ($[M + H]^+$ calcd for $C_{56}H_{82}N_5O_9$: 968.6107).

IR, NMR and MS characterization of LENK-SQ-Dig: IR (neat, cm^{-1}): ν 3297, 3068, 2958, 2924, 2851, 1653, 1516, 1443, 1260, 1142, 1099, 1020, 799, 699, 583. ^{1}H NMR (400 MHz, MeOD) δ: 7.30–7.22 (m, 4H, $2H_{Ar\text{-}ortho}$ Phe, $2H_{Ar\text{-}meta}$ Phe), 7.19 (m, 1H, $H_{Ar\text{-}para}$ Phe), 7.06 (d, 2H, $H_{Ar\text{-}ortho}$ Tyr, *J* = 8.5 Hz), 6.71 (d, 2H, $H_{Ar\text{-}meta}$ Tyr, *J* = 8.5 Hz), 5.20–5.05 (m, 5H, *H*C=C(CH_3)), 4.65 (dd, 1H, CH Phe, *J* = 4.7 Hz, *J* = 9.4 Hz), 4.57 (dd, 1H, CH Tyr, *J* = 6.1 Hz, *J* = 8.3 Hz), 4,40 (m, 1H, CH Leu), 4.17-3.85 (m, 6H, 2 CH_2 Diglycolyl, CH_2-O SQ), 3.90-3.72 (m, 4H, 2 CH_2 Gly), 3.20 (dd, 1H, C*Ha*Hb Phe , *J* = 4.7 Hz, *J* = 14.0 Hz), 3.11 (dd, 1H, C*Ha*Hb Tyr, *J* = 6.1 Hz, *J* = 13.9 Hz), 3.00-2.89 (m, 2H, CHa*Hb* Phe, CHa*Hb* Tyr), 2.14-1.93 (m, 19H, 9 CH_2 SQ, C*Ha*Hb-CH_2-O SQ), 1.74 (m, 1H, CHa*Hb*-CH_2-O SQ), 1.71–1.54 (m, 21H, CH_2 Leu, C*H*(CH_3)$_2$, 6 CH_3 SQ), 0.94 (d, 3H, CH_3 Leu, *J* = 6.2 Hz), 0.91 (d, 3H, CH_3 Leu, *J* = 6.2 Hz). ^{13}C NMR (75 MHz, MeOD) δ: 176.8 (CONH), 174.2 (CONH), 173.4 (CONH), 172.2 (O-*C*O-CH_2), 172.0 (CONH), 171.3 (CONH), 157.5 ($C_{Ar\text{-}para}$ Tyr), 138.5 ($C_{Ar\ Phe}$), 135.9 (3 HC= *C*(CH_3)), 134.8 (HC=*C*(CH_3)), 132.0 (HC=*C*(CH_3)), 131.4 ($2CH_{Ar\text{-}ortho}$ Tyr), 130.4 ($2CH_{Ar\text{-}ortho}$ Phe), 129.4 ($2CH_{Ar\text{-}meta}$ Phe), 128.6 (C_{Ar} Tyr), 127.7 ($CH_{Ar\text{-}para}$ Phe), 126.3 (H*C*=C(CH_3)), 125.6 (2 H*C*=C(CH_3)), 125.5 (H*C*=C(CH_3)), 125.4 (H*C*=C(CH_3)), 116.3 ($2CH_{Ar\text{-}meta}$ Tyr), 71.5 (O-CH_2-O), 69.4 (CO-*C*H_2-O), 65.9 (CH_2-CH_2-*C*H_2-O), 56.2 (CH Tyr), 56.0 (CH Phe), 52.3 (CH Leu), 44.0 (CH_2 Gly), 43.4 (CH_2 Gly), 41.7 (*C*H_2-CH(CH_3)$_2$ Leu), 38.6 (CH_2Phe), 37.9 (CH_2 Tyr), 36.8 (*C*H_2-CH_2-CH_2-O), 29.2 (CH_2 SQ), 27.8 (2 CH_2 SQ), 27.6 (3 CH_2 SQ), 25.9 (*C*H(CH_3)$_2$ Leu, CH_3 SQ), 23.4 (CH_3 Leu), 22.0 (CH_3 Leu), 17.8 (CH_3 SQ), 16.2 (2 CH_3 SQ), 16.0 (2 CH_3 SQ). HRMS (-ESI): *m/z* 1038.61572 ($[M - H]^-$ calcd for $C_{59}H_{84}N_5O_{11}$: 1038.61618).

IR, NMR and MS characterization of LENK-SQ-Am: IR (neat, cm^{-1}): ν 3303, 2957, 2925, 2856, 1711, 1697, 1543, 1516, 1440, 1282, 1241, 1213, 828, 671. ^{1}H NMR (400 MHz, MeOD) δ: 7.31–7.22 (m, 4H, $2H_{Ar\text{-}ortho}$ Phe, $2H_{Ar\text{-}meta}$ Phe), 7.18 (m, 1H, $H_{Ar\text{-}para}$ Phe), 7.05 (d, 2H, $H_{Ar\text{-}ortho}$ Tyr, *J* = 8.5 Hz), 6.71 (d, 2H, $H_{Ar\text{-}meta}$ Tyr, *J* = 8.5 Hz), 5.19–5.05 (m, 5H, *H*C=C(CH_3)), 4.68 (dd, 1H, CH Phe, *J* = 4.9 Hz, *J* = 9.2 Hz), 4.50-4.39 (m, 2H, CH Tyr, CH Leu), 3.87-3.67 (m, 4H, 2 CH_2 Gly), 3.20 (dd, 1H, C*Ha*Hb Phe , *J* = 4.9 Hz, *J* = 14.0 Hz), 3.07-2.93 (m, 2H, CHa*Hb* Phe, C*Ha*Hb Tyr), 2.85 (dd, 1H, CHa*Hb* Tyr, *J* = 8.2 Hz, *J* = 13.8 Hz), 2.31 (m, 2H, CH_2-CH_2-CO), 2.18 (m, 2H, CH_2-CH_2-CO), 2.13-1.88 (m, 16H, 8 CH_2 SQ), 1.73-1.53 (m, 21H, CH_2 Leu, C*H*(CH_3)$_2$ Leu, 6 CH_3 SQ), 0.94 (d,

3H, CH_3 Leu, *J* = 6.2 Hz), 0.91 (d, 3H, CH_3 Leu, *J* = 6.2 Hz). ^{13}C NMR (75 MHz, MeOD) δ: 176.2 (CO_2H), 175.8 (CONH), 174.7 (CONH), 173.3 (CONH), 172.0 (CONH), 171.2 (CONH), 157.4 ($C_{Ar\text{-}para}$ Tyr), 138.4 ($C_{Ar\ Phe}$), 136.0 (2 HC=*C*(CH_3)), 135.8 (HC=*C*(CH_3)), 134.7 (HC=*C*(CH_3)), 132.0 (HC=*C*(CH_3)), 131.3 (2$CH_{Ar\text{-}ortho}$ Tyr), 130.4 (2$CH_{Ar\text{-}ortho}$ Phe), 129.4 (2$CH_{Ar\text{-}meta}$ Phe), 128.9 (C_{Ar} Tyr), 127.7 ($CH_{Ar\text{-}para}$ Phe), 126.2 (H*C*=C(CH_3)), 125.6 (H*C*=C(CH_3)), 125.5 (H*C*=C(CH_3)), 125.5 (2 H*C*=C(CH_3)), 116.3 (2$CH_{Ar\text{-}meta}$ Tyr), 56.9 (CH Tyr), 55.9 (CH Phe), 52.3 (CH Leu), 43.9 (CH_2 Gly), 43.3 (CH_2 Gly), 41.7 (*C*H_2-CH(CH_3)$_2$ Leu), 38.7 (CH_2Phe), 37.9 (CH_2 Tyr), 36.5 (*C*H_2-CH_2-CO), 35.8 (CH_2-*C*H_2-CO), 29.2 (3 CH_2 SQ), 27.8 (4 CH_2 SQ), 27.5 (2 CH_2 SQ), 25.9 (*C*H(CH_3)$_2$ Leu, CH_3 SQ), 23.4 (CH_3 Leu), 21.9 (CH_3 Leu), 17.7 (CH_3 SQ), 16.2 (2 CH_3 SQ), 16.1 (CH_3 SQ), 16.0 (CH_3 SQ). HRMS (-ESI): *m/z* 936.5826 ([M - H]$^-$ calcd for $C_{55}H_{78}N_5O_8$: 936.5845).

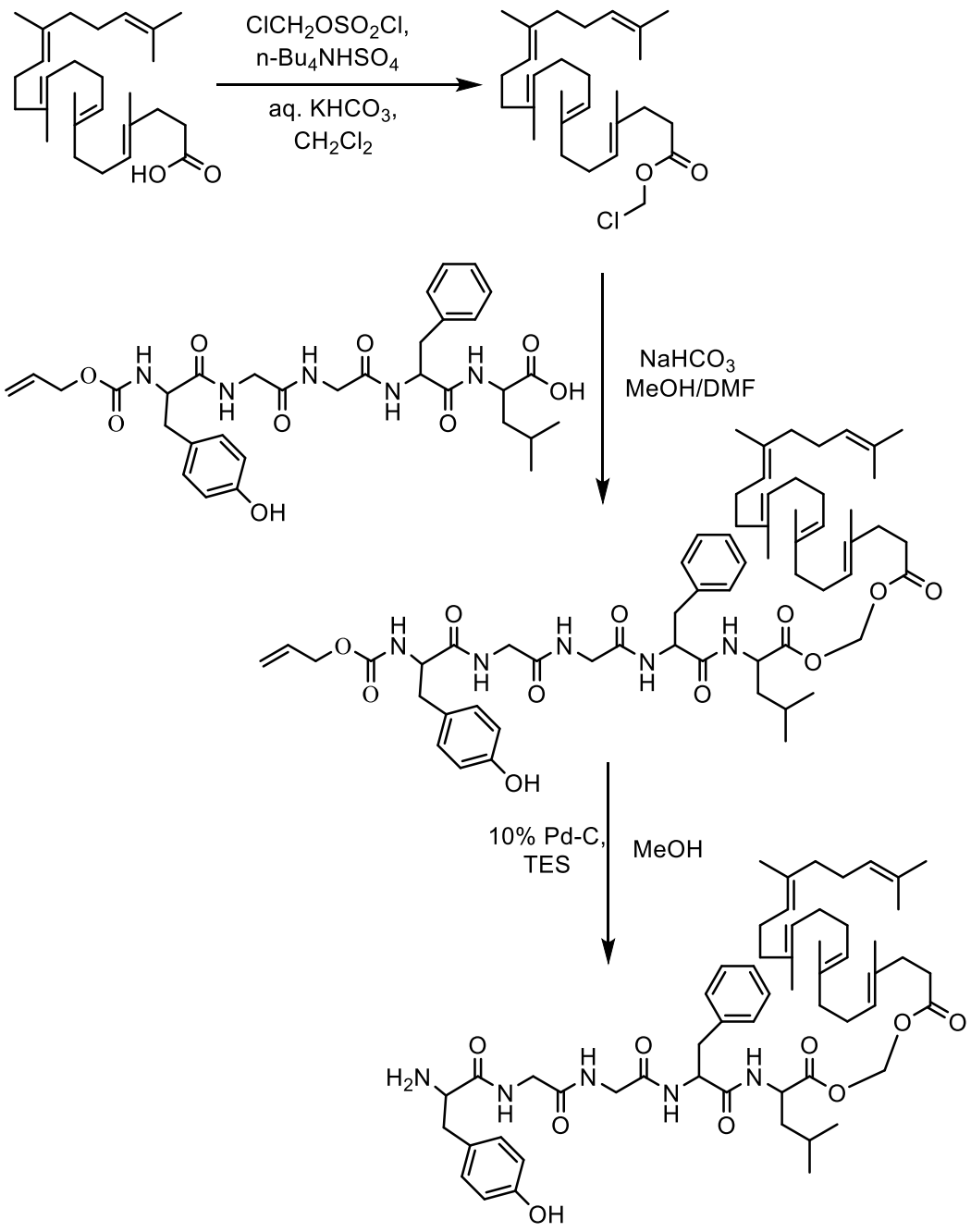

Fig. S1. Synthesis of LENK-SQ-Diox.

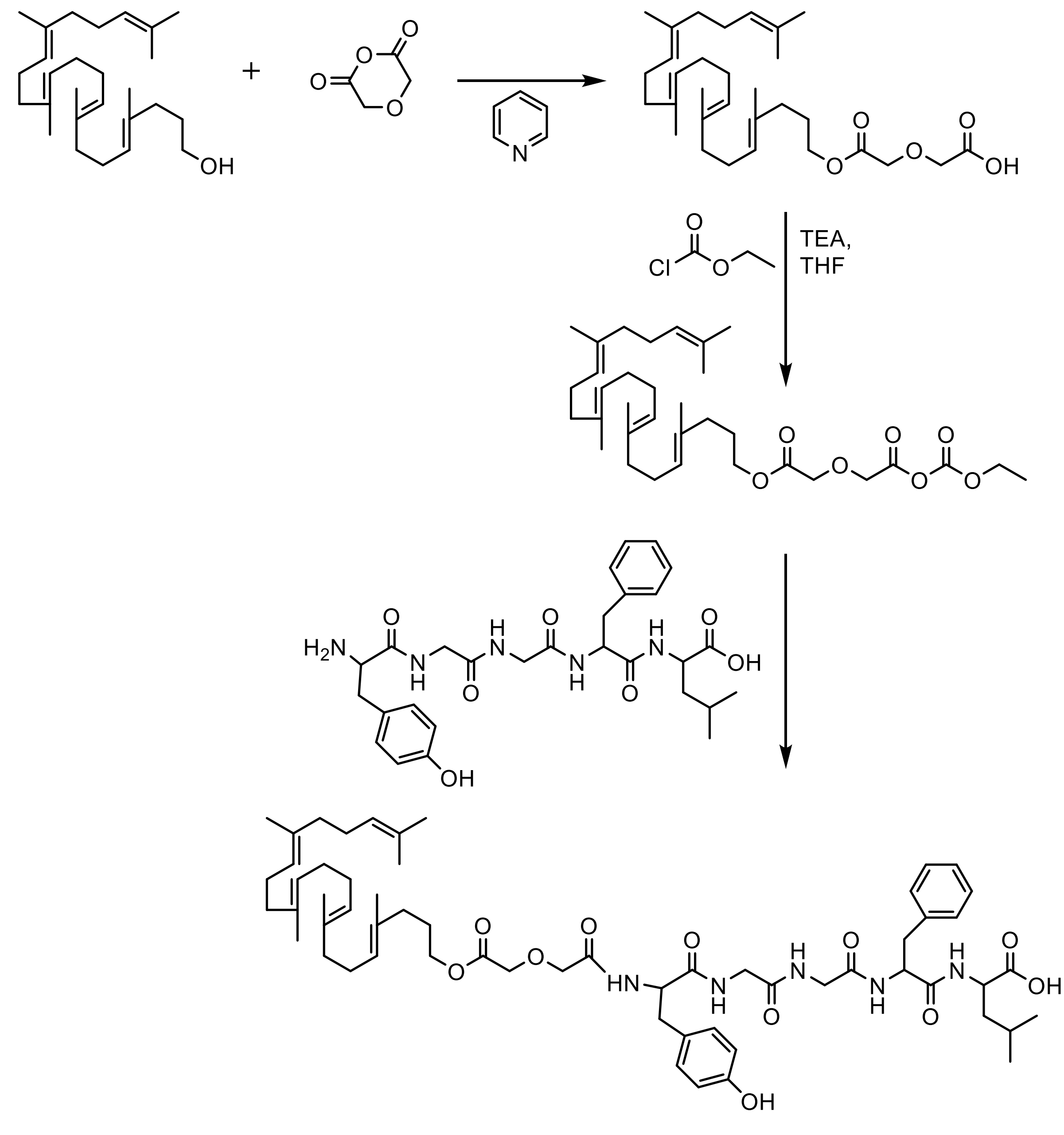

Fig. S2. Synthesis of LENK-SQ-Dig.

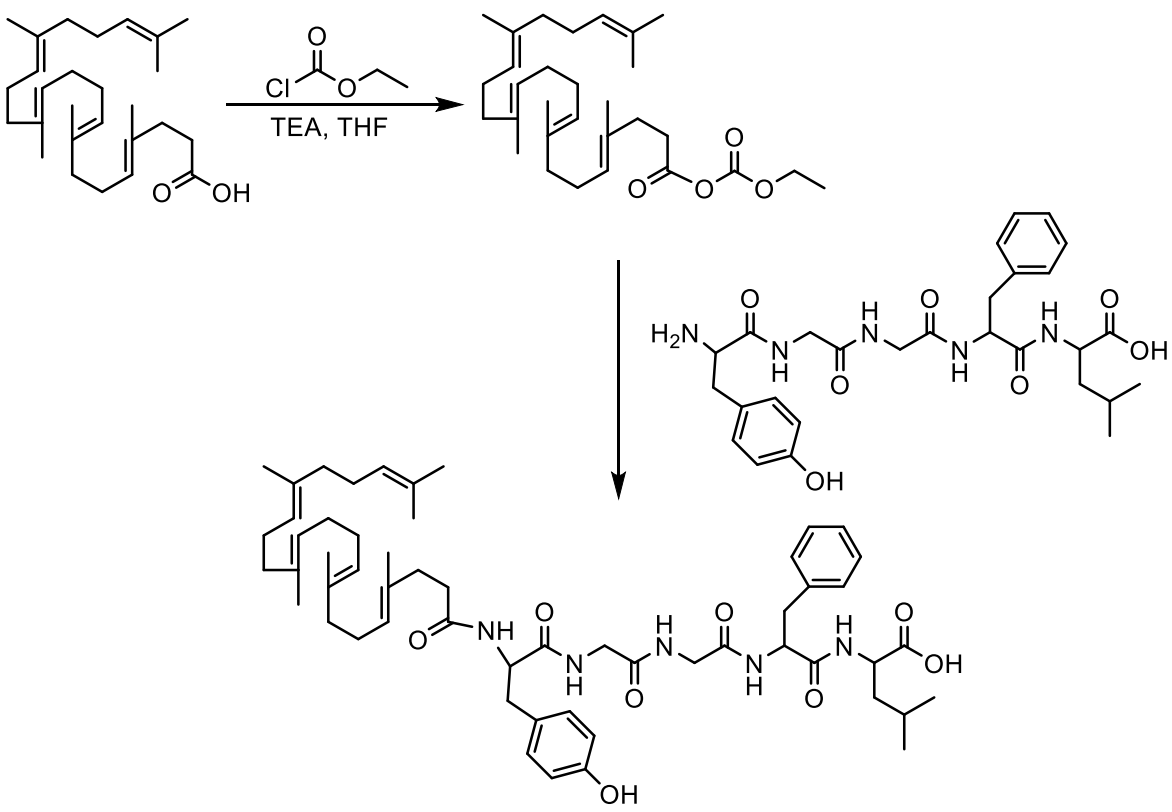

Fig. S3. Synthesis of LENK-SQ-Am.

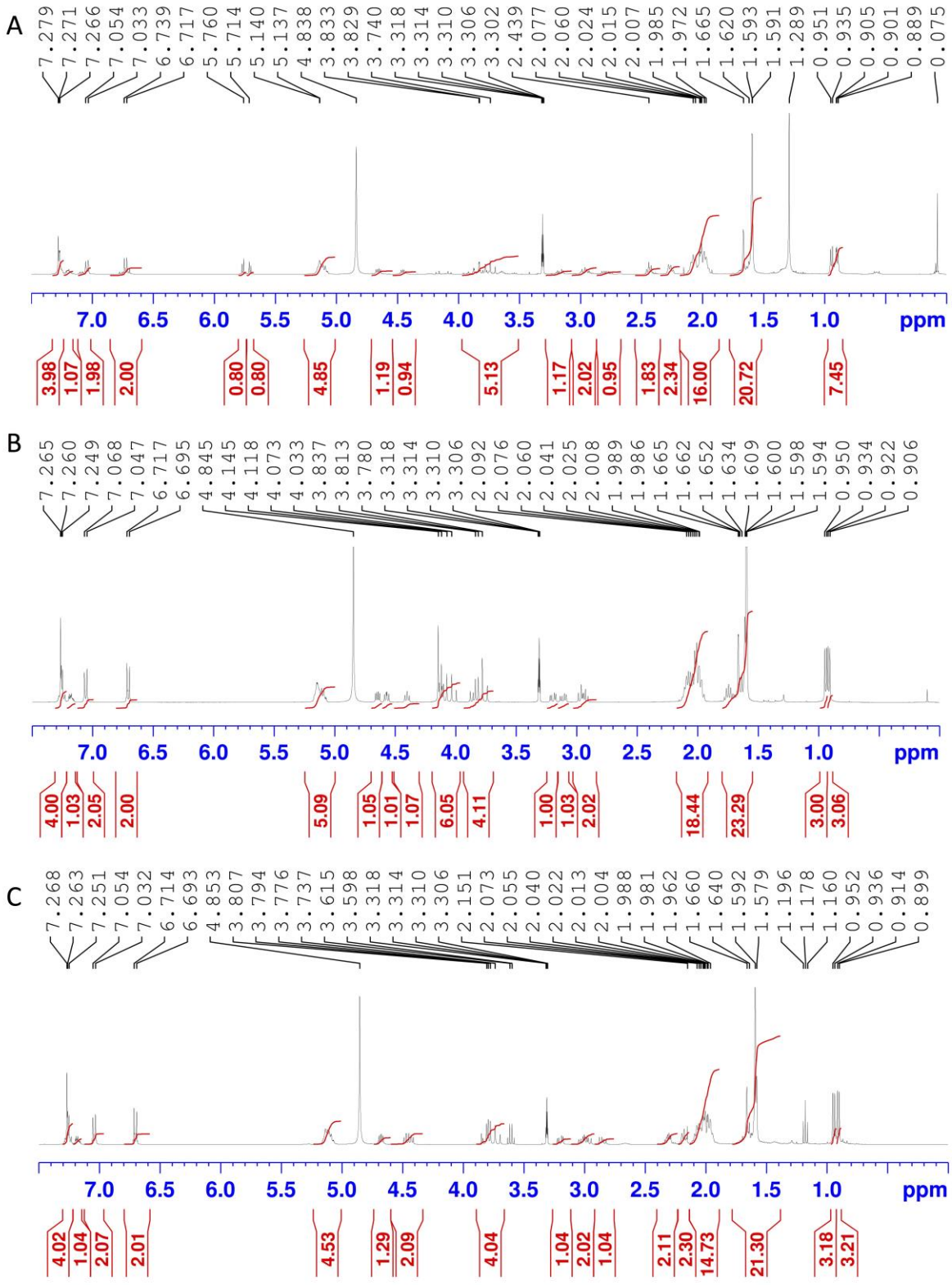

Fig. S4. ^{1}H spectrum of LENK-SQ bioconjugates. (A) LENK-SQ-Diox, **(B)** LENK-SQ-Dig, and **(C)** LENK-SQ-Am.

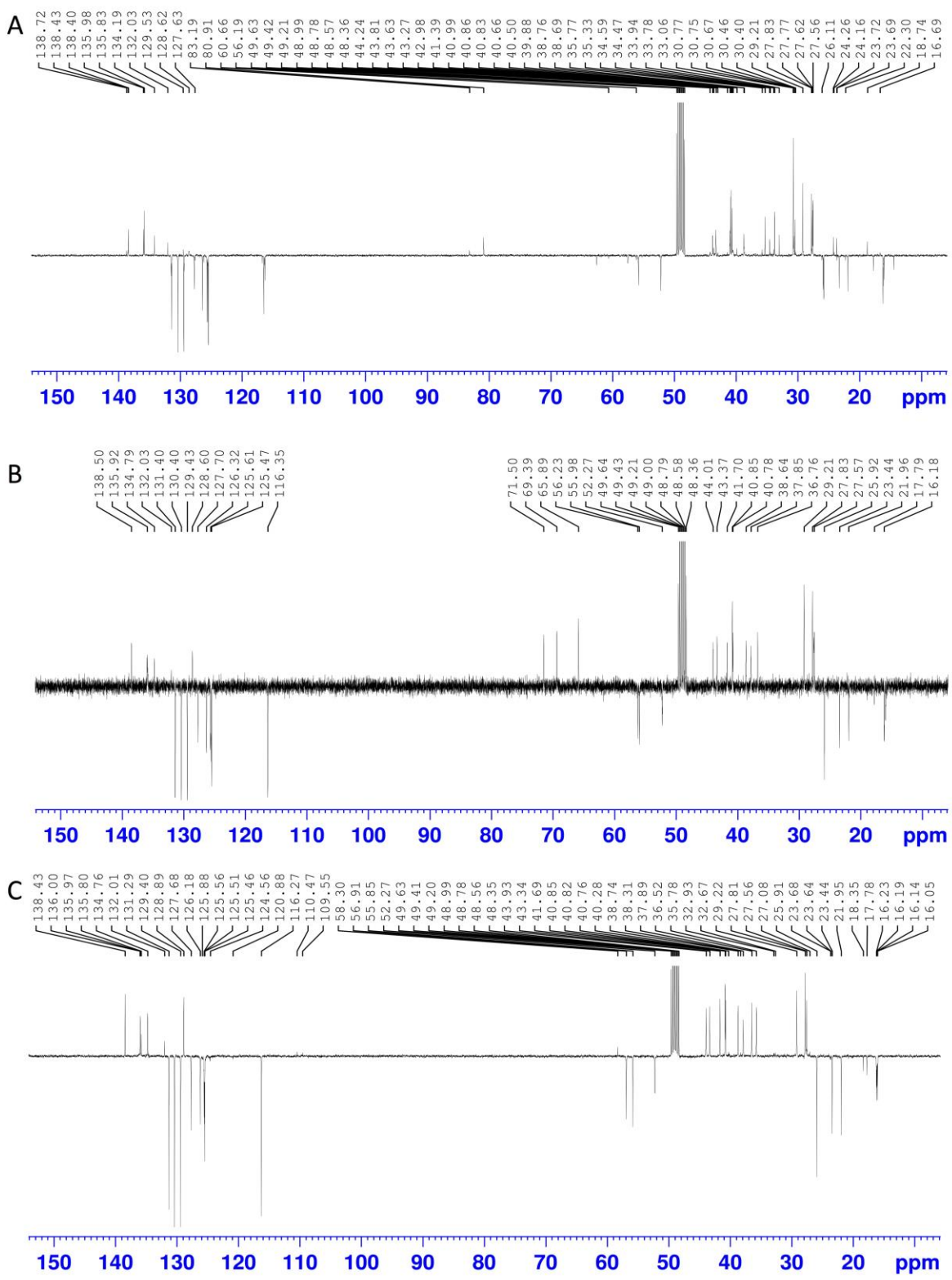

Fig. S5. ^{13}C spectrum of LENK-SQ bioconjugates. (A) LENK-SQ-Diox, **(B)** LENK-SQ-Dig, and **(C)** LENK-SQ-Am.

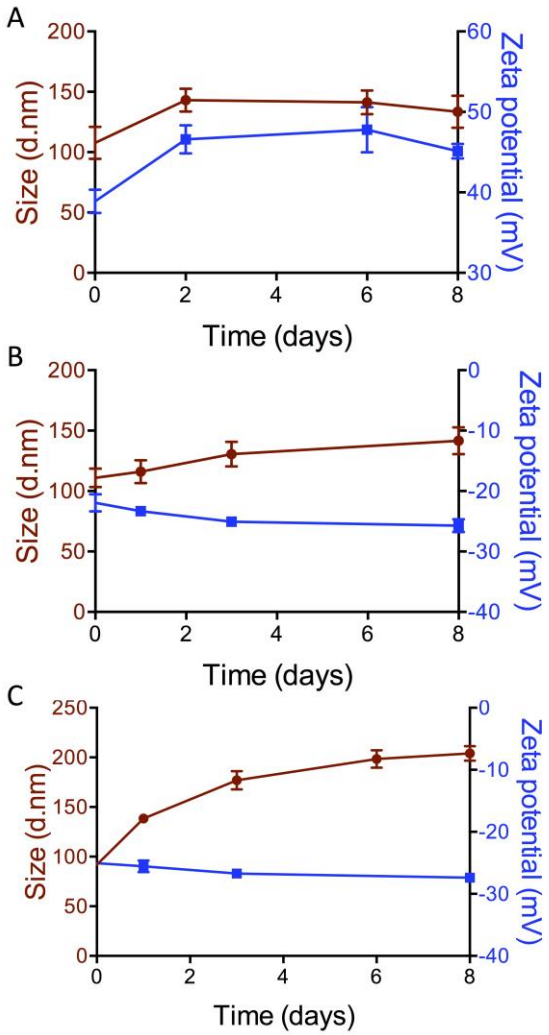

Fig. S6. Size and zeta potential of LENK-SQ NPs kept at +4°C. (A) LENK-SQ-Diox NPs, **(B)** LENK-SQ-Dig NPs and **(C)** LENK-SQ-Am NPs. Results of three independent preparations are presented as mean ± SEM.

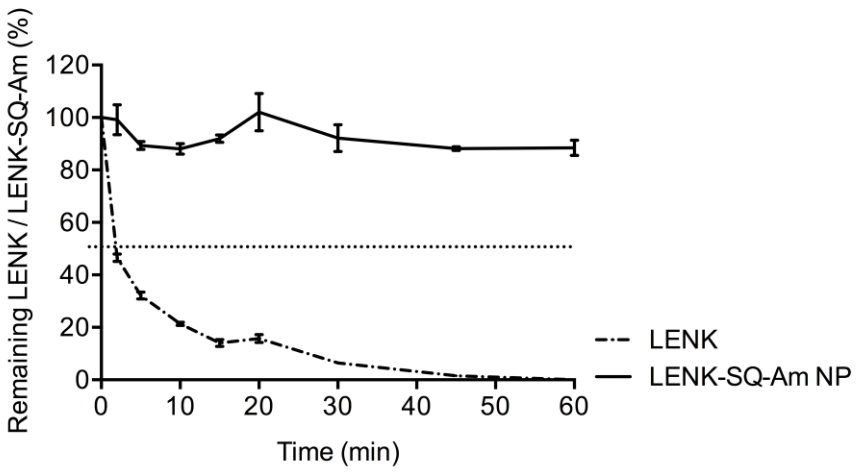

Fig. S7. Hydrolysis of LENK or LENK-SQ-Am NPs in the presence of serum. 300 μL of LENK-SQ-Am NPs (2 mg/mL, 2 mmol) or LENK (1.15 mg/mL, 2 mmol) were incubated in 900 μL mouse serum, and samples were collected at different times for HPLC analysis. The LENK-SQ bioconjugate was unaltered during the course of the experiment, whereas free LENK was rapidly metabolized.

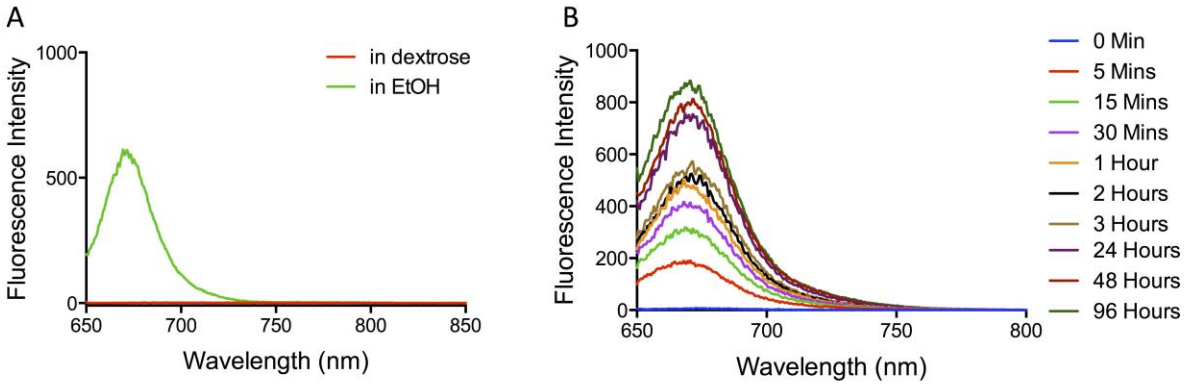

Fig. S8. In vitro colloidal stability of LENK-SQ-Am NPs in mouse serum. (A) Controls: When diluted in 5% dextrose LENK-SQ-Am NPs remained assembled (DiD: reporter dye; DiR: quencher). They disassembled in ethanol; **(B)** LENK-SQ-Am NPs (DiD: reporter dye; DiR: quencher) incubated in mouse serum (1:4). The fluorescence emission was measured to assess the progressive disassembly of the nanoparticles.

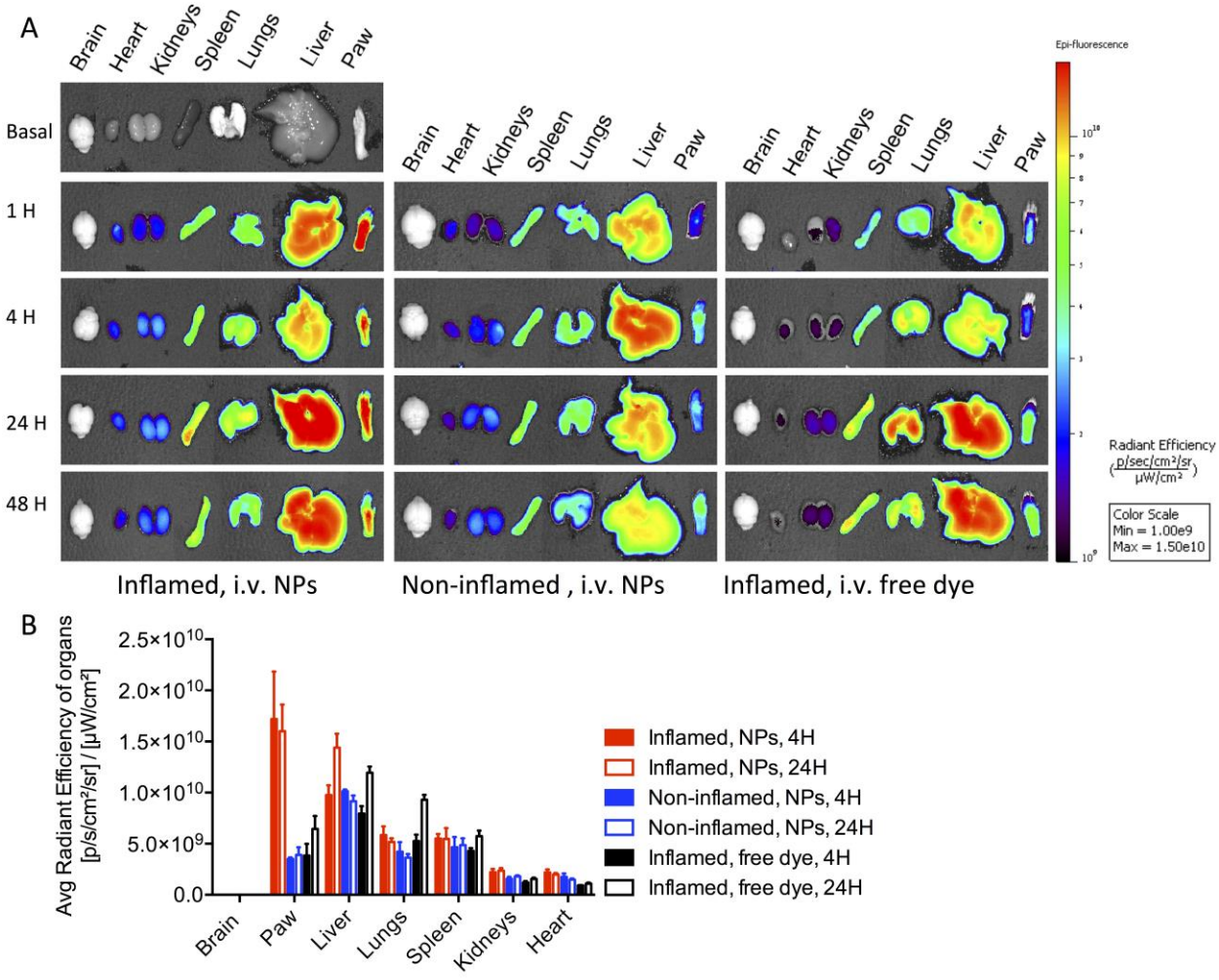

Fig. S9. Biodistribution of fluorescent LENK-SQ-Am NPs or control fluorescent dye solution in mice with or without inflamed paw. 4 h after λ-carrageenan or saline injection into the right paw, fluorescent LENK-SQ NPs or free dye were intravenously introduced into the mice. At different time points, mice were deeply anesthetized with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) before euthanasia by transcardiac perfusion of 40 ml saline (8 mL/min), until the fluid exiting the right atrium was entirely clear. Then, liver, spleen, kidneys, heart, lungs, brain, and inflamed right hind paw were excised and immediately imaged with the imager. The fluorescence emitted was quantified with Living Image software over the region of interest (ROI) with the threshold of 20%. (**A**) *Ex vivo* fluorescence imaging of the harvested brain, heart, kidneys, lungs, liver and paw from fluorescent NPs or free dye-injected SWISS mice. (**B**) Average radiant efficiency of these organs after 4 or 24 h injection of NPs or free dye.

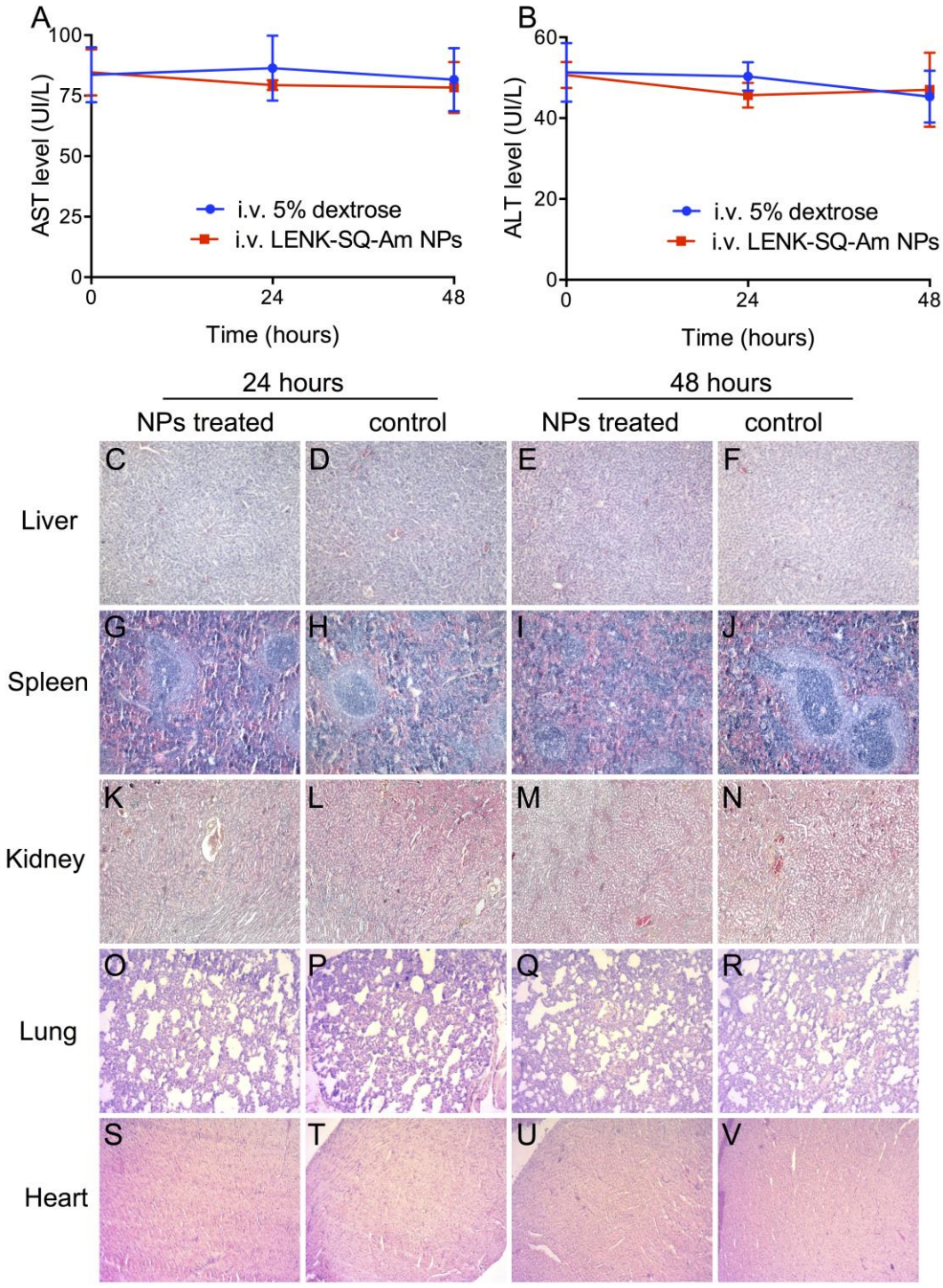

Fig. S10. Toxicity study of LENK-SQ-Am NPs upon systemic administration. LENK-SQ-Am NPs (20mg/kg) were intravenously administered in rats. The AST **(A)** and ALT **(B)** levels in plasma showed no differences compared with dextrose solution (data presented as mean UI/L ± SED, N = 3 animals per group). Histological analysis of organs after intravenous administration of LENK-SQ-Am NPs (20mg/kg) did not show any signs of cell or tissue damage at 24 h and 48h, comparatively to a control 5% dextrose solution. Liver **(C-F)**, spleen **(G-J)**, kidneys **(K-N)**, lungs **(O-R)** and heart **(S-V)**. All tissue images were analyzed by microscopy at 10× magnification except for kidneys which were at 5× magnification (Zeiss).