Electronic Supplementary Information

LDH-doped electrospun short fibers enable dual drug loading and multistage release for chemotherapy of drug-resistant cancer cells

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Experimental Section

Materials

Poly(lactic-co-glycolic acid) (PLGA, LA:GA = 50:50, Mw = 81,000 g/mol, η = 0.58) was purchased from Jinan Daigang Biomaterial Co., Ltd. (Jinan, China). Doxorubicin hydrochloride (DOX·HCl) was acquired from Beijing Huafeng Pharmaceutical Co., Ltd. (Beijing, China). Magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6H_2O$), aluminum nitrate nine hydrate ($Al(NO_3)_3 \cdot 9H_2O$), sodium hydroxide (NaOH), tetrahydrofuran (THF), N,N-dimethylformamide (DMF), and D-α-tocopherol succinate (α-TOS) were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Poly(vinyl alcohol) (PVA, Mw = 85-124 kDa) was purchased from Sigma-Aldrich (St. Louis, MO). 4',6-Diamidino-2-phenylindole (DAPI) was from Bestbio. Co., Ltd. (Shanghai, China). FITC-phalloidin was obtained from Invitrogen (Carlsbad, CA). Regenerated cellulose dialysis membranes with a molecular weight cut-off (MWCO) of 3000 were from Yuanye Biotechnology Corporation (Shanghai, China). MCF-7 cells (a human breast cancer cell line) were obtained from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). MCF-7/Adriamycin (MCF-7/ADR) cells were purchased from Fuheng Biology Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) was from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Minimum eagle's medium (MEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco (Carlsbad, CA). Cell Counting Kit-8 (CCK-8) was purchased from 7Sea Pharmatech Co., Ltd. (Shanghai, China). All chemicals were used as received. Water used in all experiments was purified using a PURIST UV Ultrapure Water System (RephiLe Bioscience, Ltd., Shanghai, China) with a resistivity higher than 18.2 MΩ cm.

Characterization Techniques

Hydrodynamic size distribution and zeta potential of the synthesized LDH and DOX/LDH were measured using a Malvern zetasizer (Nano ZS model ZEN3600, Worcestershire, UK) equipped with a standard 633-nm laser. Each sample was dispersed in water at a concentration of 1 mg/mL before analysis. Phenom XL scanning electron microscope (SEM, JEOL JSM-5600LV, Tokyo, Japan) was adopted to observe the morphology of LDH, nanofibrous membranes and short nanofibers. LDH and

short fibers were dispersed in water and deposited onto aluminum foil and then air dried. All samples were sputter coated with a gold film with a thickness of 10 nm before SEM measurements. Then, the size (diameter and/or length) distribution of each sample was analyzed using ImageJ software (https://imagej.nih.gov/ij/download.html). At least 200 LDH or nanofibers from different SEM images were measured for each sample. In order to observe the distribution of LDH inside the PLGA fibers, transmission electron microscopy (TEM) was performed using a JEOL 2010F electron microscope (Tokyo, Japan) at an operating voltage of 200 kV. An aqueous suspension of short nanofibers with a volume of 5 μL was dropped onto a carbon-coated copper grid and air dried before TEM measurements. Lambda 25 UV-vis spectrophotometer (Perkin Elmer, Boston, MA) was used to determine the absorbance of α-TOS at 280 nm and DOX at 480 nm, respectively. X-Ray diffraction (XRD) analysis was carried out using a D/max 2550 VB+/PC X-ray diffractometer (Rigaku Cop., Tokyo, Japan) with Cu Kα radiation (λ = 0.154056 nm) at 40 kV and 200 mA and a 2θ scan range of 5-90°. Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) spectra were recorded on a Thermo-Nicolet Nexus 670 FTIR spectrophotometer (Madison, WI) in a wavenumber range of 500-4000 cm^{-1}. Each sample was mixed with milled KBr crystals and pressed to form pellets before measurements.

***In Vitro* Drug Release**

In order to investigate the drug release profile of DOX and α-TOS, the *in vitro* drug release assay was conducted under different pH conditions. DOX/PLGA or DOX@LDH/α-TOS/PLGA short nanofibers (5 mg for each sample) were dispersed in 1 mL of phosphate buffer (pH = 7.4, 6.8, and 5.5, respectively) and transferred to a dialysis bag with a molecular weight cut-off of 3000. Then, the dialysis bag was tightly sealed and immersed in the corresponding buffer medium (9 mL), and the whole system was placed in a constant temperature shaker (37 °C, 160 rpm). For each sample, 5 parallel specimens were tested. At each predetermined time point, 1 mL of external releasing medium was taken out, and thereafter 1 mL of corresponding buffer medium was replenished. The cumulative drug release from DOX/PLGA and DOX@LDH/α-TOS/PLGA short nanofibers was investigated using UV-vis spectrophotometer to record the absorbance of α-TOS at 280 nm and DOX at 480 nm

based on the respective absorbance-concentration calibration curve.

Hemolytic Assay

The hemocompatibility of the prepared LDH/PLGA, DOX/PLGA and DOX@LDH/α-TOS/PLGA short nanofibers was evaluated *via* hemolysis assay according to protocols described in our previous work.[1] Human whole blood stabilized with heparin from healthy adult volunteers was kindly provided by Shanghai General Hospital (Shanghai, China) after approval by the ethical committee of the same hospital. In brief, human red blood cells (HRBCs) were obtained from fresh whole blood (1 mL) by centrifugation and washing with phosphate buffered saline (PBS), followed by diluting 30 times with PBS to establish a negative control (0.2 mL HRBC suspension, 0.8 mL PBS) and a positive control (0.2 mL HRBC suspension, 0.8 mL water), respectively.

Anticoagulant Assay

The anticoagulant property of the LDH/PLGA, DOX/PLGA, and DOX@LDH/α-TOS/PLGA short nanofibers (10 mg) was determined by a kinetic clotting time method described in the previous literature.[1] In brief, the as-prepared LDH/PLGA, DOX/PLGA, and DOX@LDH/α-TOS/PLGA short nanofibers were put into individual well of a 12-well tissue culture plate. Cover slips without nanofibers were used as control. Then, fresh human blood (20 μL) was dropped onto the surface of the nanofiber samples or the cover slips, respectively, followed by adding 10 μL of $CaCl_2$ solution (0.2 M) to each blood drop and incubating the samples at 37°C for a predetermined periods of time (5, 10, 40, and 60 min, respectively). After that, 5 mL of water was put into each well carefully and incubated at 37 °C for 5 min. The concentration of hemoglobin in water was measured by monitoring the absorbance at 540 nm using a UV–vis spectrophotometer.

Cytotoxicity Assay

Cytotoxicity assays were performed to confirm the cytocompatibility of drug-free LDH/PLGA short nanofibers, as well as the anticancer activity of drug-loaded short nanofibers. The cytotoxicity of short nanofibers against both MCF-7 and MCF-7/ADR cells was assessed by CCK-8 assay. Briefly, the MCF-7 or MCF-7/ADR cells were seeded in 96-well plates with a cell density of 1×10^4 cells/well.

After incubated overnight, the cells were treated with free DOX, LDH/PLGA, DOX/PLGA, α-TOS/PLGA, or LDH@DOX/α-TOS/PLGA short nanofibers (100 μL, in complete culture medium) at 37 °C for 24 h. The DOX and α-TOS concentrations is controlled with a range of 5-40 μg/mL. Among them, LDH/PLGA and α-TOS/PLGA short nanofibers had the same LDH or α-TOS concentration as that of LDH@DOX/α-TOS/PLGA short nanofibers, while free DOX, DOX/PLGA and LDH@DOX/α-TOS/PLGA had identical DOX equivalent. Afterwards, each well was added with 100 μL FBS-free medium containing 10% (v/v) of CCK-8 solution (10 μL CCK8 stock solution plus 90 μL FBS-free medium) and the cells were incubated for another 4 h. Then, the Thermo Scientific Multiskan MK3 ELISA reader (Waltham, MA) was used to record the absorbance of each well at 450 nm.

Cellular Uptake Assay

Cellular uptake of LDH@DOX/α-TOS/PLGA short nanofibers were investigated through flow cytometry and confocal laser scanning microscopy. In brief, MCF-7 cells and MCF-7/ADR cells were seeded in 12-well plates with a density of 1×10^5 cells/well each with 1 mL respective cell medium. After incubation for 24 h, the culture medium was replaced with fresh medium containing DOX@LDH/α-TOS/PLGA short nanofibers with a DOX concentration ranging from 5 to 20 μg/mL. After co-incubation for 3 h, the medium was discarded and the cells were rinsed with PBS for 3 times, trypsinized, centrifuged and resuspended in 300 μL PBS. The intensity of DOX fluorescence was measured using a Becton Dickinson FACScan flow cytometry (BD Biosciences, Franklin Lakes, NJ).

In addition, the MCF-7/ADR cells were seeded into each confocal dish with coverslip at a density of 5×10^4 cells/well (2 mL medium for each well) and incubated overnight, and then the cells were treated with 500 μL fresh medium containing DOX@LDH/α-TOS/PLGA short nanofibers with different final DOX concentrations (5-20 μg/mL) for another 3 h. The culture medium was removed and cells in each well were washed 3 times with PBS. Then, the cells were fixed with glutaraldehyde (2.5%) for 30 min at 4 ^{o}C, and the cell cytoskeleton and nucleus were stained with FITC-phalloidin and DAPI, respectively according to the literature[2] before confocal microscopic imaging.

Statistical analysis

All experimental data were represented as the mean ± standard deviation through at least three parallel sample evaluations. One-way analysis of variance (ANOVA) statistical method was used to analyze all the experimental results through IBM SPSS Statistic 25 software. A p value of 0.05 was selected as a significance level, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Hydrodynamic size, zeta potential and PDI of LDH and DOX@LDH.

Materials	Hydrodynamic size (nm)	ξ-potential (mV)	Polydispersity Index (PDI)
LDH	80.5 ± 15.3	26.1 ± 1.5	0.2 ± 0.02
DOX@LDH	120.3 ± 10.7	10.5 ± 2.3	0.3 ± 0.02

Table S2. Hemolysis percentages of HRBCs exposed to LDH/PLGA, DOX/PLGA, and DOX@LDH/α-TOS/PLGA short fibers (2, 4, and 6 mg/mL, respectively) for 2 h.

Short fibers	Hemolytic percentage (%)		
	2 mg/mL	4 mg/mL	6 mg/mL
LDH/PLGA	3.5 ± 0.03	1.4 ± 0.01	0.2 ± 0.01
DOX/PLGA	4.2 ± 0.03	2.7 ± 0.01	0.9 ± 0.01
DOX@LDH/α-TOS/PLGA	3.0 ± 0.01	1.5 ± 0.02	0.1 ± 0.01

Table S3. The half maximal inhibition concentration (IC_{50}) of the free DOX and DOX@LDH/α-TOS/PLGA short nanofibers cultured with MCF-7 or MCF-7/ADR cells.

Cells	IC_{50}（μg/mL）	
	DOX	DOX@LDH/α-TOS/PLGA
MCF-7	6.59	27.18
MCF-7/ADR	25.74	106.13

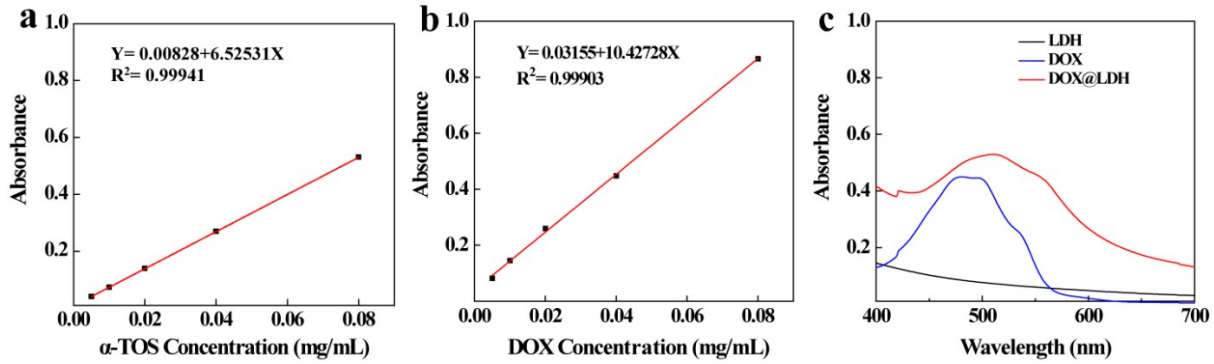

Figure S1. (a) Absorbance-concentration calibration curve of α-TOS at a wavelength of 280 nm. (b) Absorbance-concentration calibration curve of DOX at a wavelength of 480 nm. (c) UV-vis spectra of LDH, free DOX, and DOX@LDH in aqueous solution. The standard curve of α-TOS is $Y = 0.00828 + 6.52531X$, $R^2 = 0.99941$. The standard curve of free DOX is $Y = 0.03155 + 10.42728X$, $R^2 = 0.99903$.

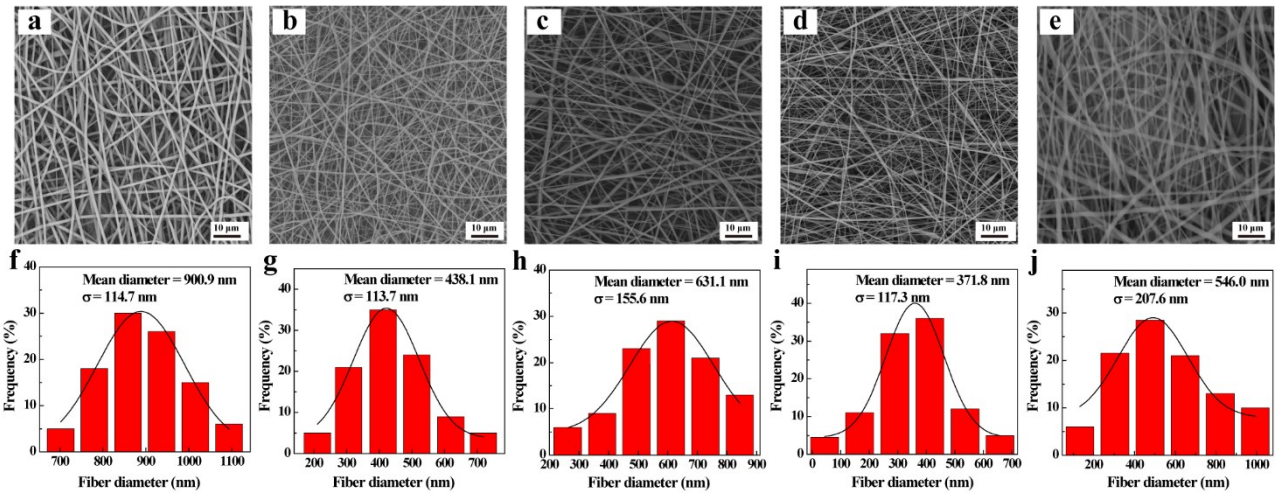

Figure S2. SEM images and diameter distribution histograms of electrospun (a,f) PLGA, (b,g) LDH/PLGA, (c,h) DOX/PLGA, (d,i) α-TOS/PLGA, and (e,j) DOX@LDH/PLGA nanofibers.

The surface morphology of the PLGA, LDH/PLGA, DOX/PLGA, α-TOS/PLGA, and DOX@LDH/α-TOS/PLGA nanofibers were observed via SEM (**Fig. S2a-e**). With the easy spinnability of PLGA, the incorporation of DOX, α-TOS, or DOX@LDH does not seem to significantly alter the uniform and smooth fibrous morphology of PLGA nanofibers.[3-5] The diameters

of the PLGA (**Fig. S2a**), LDH/PLGA (**Fig. S2b**), DOX/PLGA (**Fig. S2c**), α-TOS/PLGA (**Fig. S2d**), and DOX@LDH/PLGA (**Fig. S2e**) nanofibers were estimated to be 900.9 ± 114.7 nm (**Fig. S2f**), 438.1 ± 113.7 nm (**Fig. S2g**), 631.1 ± 155.6 nm (**Fig. S2h**), 371.8 ± 117.3 nm (**Fig. S2i**), and 546 ± 207.6 nm (**Fig. S2j**), respectively. The composite LDH/PLGA, DOX/PLGA, α-TOS/PLGA, and DOX@LDH/PLGA nanofibers have smaller diameters than pure PLGA nanofibers, presumably due to the increased solution conductivity or viscosity, which was caused by the introduction of LDH, DOX, α-TOS or DOX@LDH species in the electrospinning solution.

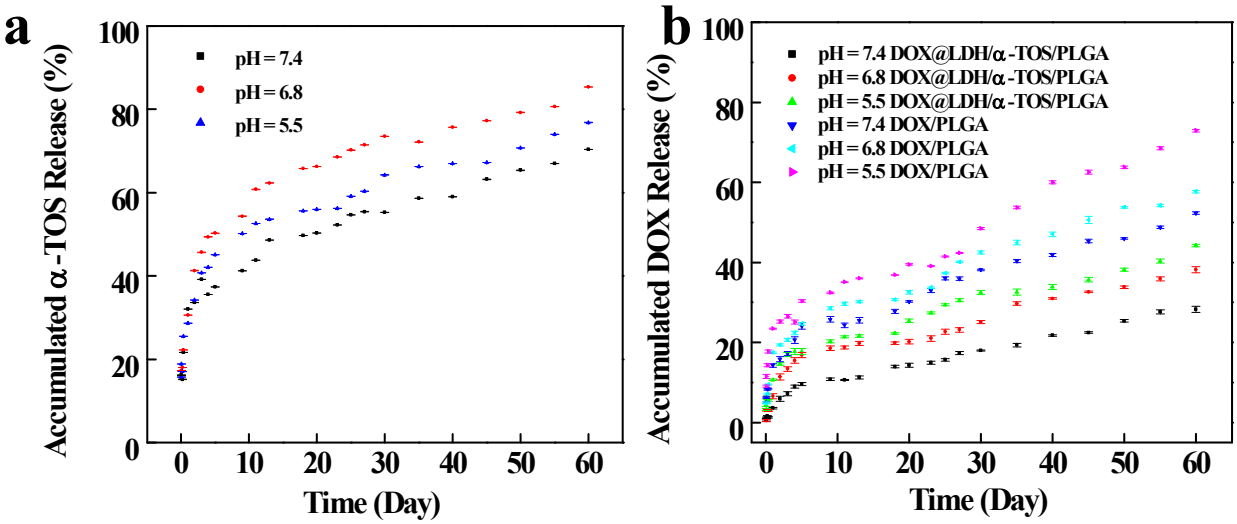

Figure S3. *In vitro* release of α-TOS (a) and DOX (b) from the DOX/PLGA or DOX@LDH/α-TOS/PLGA short nanofibers with the same α-TOS or DOX amount.

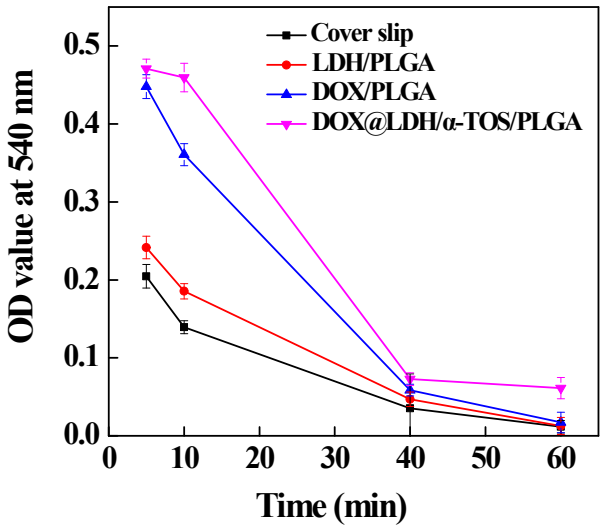

Figure S4. Optical density (OD) values at 540 nm for anticoagulant assay of cover slips, LDH/PLGA, DOX/PLGA, and DOX@LDH/α-TOS/PLGA short nanofiber at different time intervals.

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