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Moisture resistant and biocompatible CD-MOFs nanoparticles *via* cholesterol shielding

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Experimental Section

Materials and reagents

The γ-cyclodextrin was obtained from MaxDragonbiochem Ltd. Potassium hydroxide (KOH), cetyltrimethyl ammonium bromide (CTAB), 4-dimethylamino pyridine(≥99%, DMAP), isopropanol, ethanol (EtOH), acetone, dichloromethane (DCM) and N, N-Dimethylformamide (DMF) were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd (Beijing, China). Cholesterol (CHS) succinate (⩾97%) and 1-ethyl-3-3-dimethylaminopropylcarbodiimide (≥98%, EDC) was purchased from TCI chemical company (Shanghai, China). Doxorubicin (>99.5% purity) were purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, China). All cell culture reagents were obtained from Invitrogen (Carlsbad, USA).All other chemicals were of analytical grade and used without further purification.

Synthesis of nanosizedγ-CD-MOFs

The γ-CD (162 mg, 0.125 mmol) was mixed with 8equivalent moles of KOH (56 mg, 1 mmol) in 5 mL aqueous solution. The solutionwas filtered through a 0.45 μm filter membrane into a glass tube, and 0.5 mL ofMeOH was added, followed by vapor diffusion of MeOH into the solution at 50°C.After 6 hrs, the supernatant was transferred into another glass tube with addition ofCTAB (8 mg·mL-1) and the solution was incubated at room temperature overnight.To obtain the much smaller crystals of 200

nm, an equivalent volume of MeOH as that ofthe supernatant was premixed with CTAB. The precipitates were washed with isopropanol and dried at 37°C overnight[1].

Synthesis of CD-MOF-CHS

A facile procedure was set upfor the CHSattachment to the surface of CD-MOF. Briefly, 1g of CD-MOF was added to 30 mL of DMF in a 250 mL clean and dry round bottom flask following the addition of130mg of CHS hydrogen succinate (in excesswith respect to CDs on the CD-MOFs surface), 76 mg of EDC (1.5 equiv to CHS) and 32mg of DMAP (1 equiv to CHS). The reaction mixture was continuously stirred for 24hrs at60°C. After reaction completion, the reaction mixturewas cooled down to room temperature and the precipitatedparticleswerethoroughly washed with ethanol and acetone to remove catalyst and unreacted CHS.

Characterization

The nanoparticle samples were analyzed by FTIR spectroscopy using a Thermo-scientific system Nicolet™ iS™5 FT-IR Spectrophotometer in the region of 600 to 4000 cm^{-1}. The IR spectrum of CHS and CD-MOFwere also recorded as controls to compare with that from CD-MOF-CHS for structure confirmation (Figure S-1)

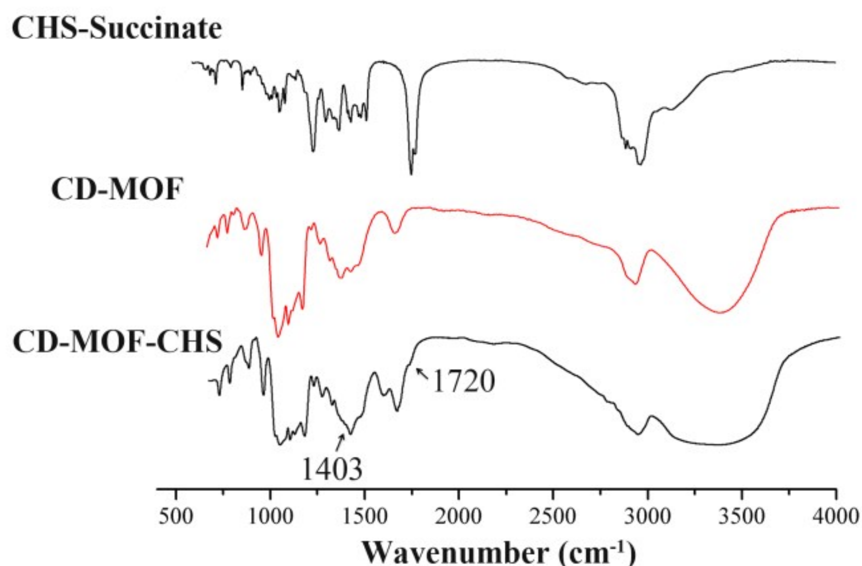

Figure S-1.FTIR spectra of CHSsuccinate, CD-MOF and CD-MOF-CHS in comparison.

Raman spectra were recorded withthe Thermo Scientific DXR Raman microscope equipped with a 532nm laser. Raman scans range from 3500 to 50 cm^{-1}. The samples

wereanalyzed directly in a glass sheet using 10 mW laser power and 50 μmpinhole spectrograph aperture. Calibration of the instrument wasperformed using a polystyrene film standard.

Morphological characterizations of all samples were conducted using a scanning electron microscope (SEM, S-3400N, Hitachi). The specimens were immobilized on a metal stub with double-sided adhesive tape and coated with a thin gold film, then observed under definite magnification. Considering the high regularity of γ-CD-MOF crystals and the value of length: width: height ≈ 1, the size of γ-CD-MOFs was measured with the Image Pro Plus 6.0 software combined with the SEM images.The crystallinity of the samples was characterized by X-ray powder diffraction (XRPD) analysis. Diffraction patterns of the prepared γ-CD-MOF and CD-MOF-CHS crystals were detected with a Bruker D8 Advance diffractometer (Bruker, Germany) at ambient temperature, with tube voltage of 40 kV, tube current of 40 mA in a stepwise scan mode (8 ° min^{-1}). Diffraction data for the thermal and chemical stability samples were collected on an X/Pert Pro 3040/60 diffractometer (PANalytical, Holland). All the samples were irradiated with monochromatizedCuKα radiation and analyzed over a 2θ angle range of 3-40 °.

Nitrogen adsorption-desorption isotherm was measured with a liquid nitrogen bath (-196 °C) using a porosimeter (TriStar 3000 V6.05 A, USA). In order to remove the interstitial solvents, the samples were activated by immersing in DCM for three days and dried under vacuum at 50 °C for 12 hrs. Known amounts of samples (e. g. 150-200 mg) were loaded into the BET sample tubes and degassed under vacuum (10-5 Torr) at 100 °C for 5 hrs. BET model was applied to measure the specific surface areas of the prepared samples.

The thermo profiles were recorded using a differential scanning calorimeter (DSC 822, Mettler Toledo, Switzerland). About 2.0 mg of each sample was heated in a pierced aluminum pan from 50 to 300 at heating rate of 10°C/min. Thermal data analysis of the DSC thermal profiles was conducted using the Mettler Toledo STAR system software. Thermogravimetric analysis (TGA) of γ-CD-MOF crystals was performed using a thermal analysis system (NETZSCH 209F3 240-20-382-L, USA) at a heating rate of

10 °C·min^{-1} under nitrogen. Samples were weighed (approximately 5 mg) in a hanging aluminum pan and the weight loss percentage of the samples was monitored from 30 to 400°C.

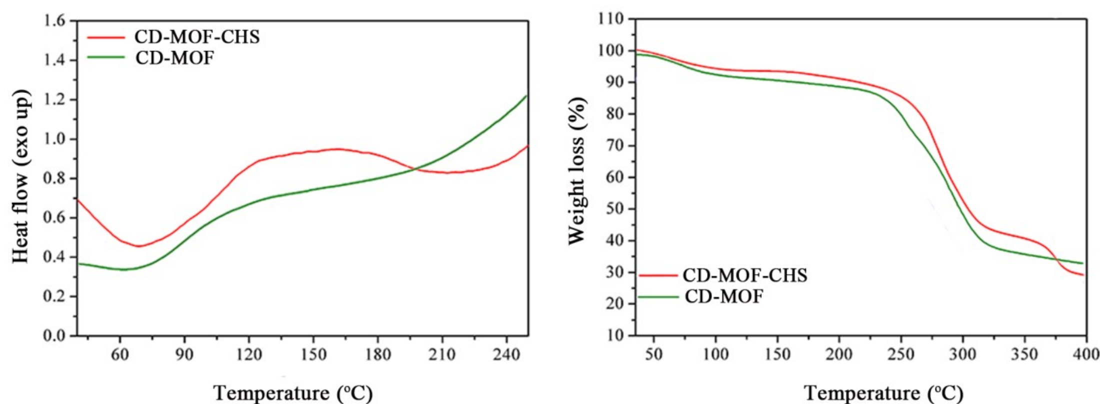

Figure S-2. The DSC and TGA comparison of CD-MOF and CD-MOF-CHS

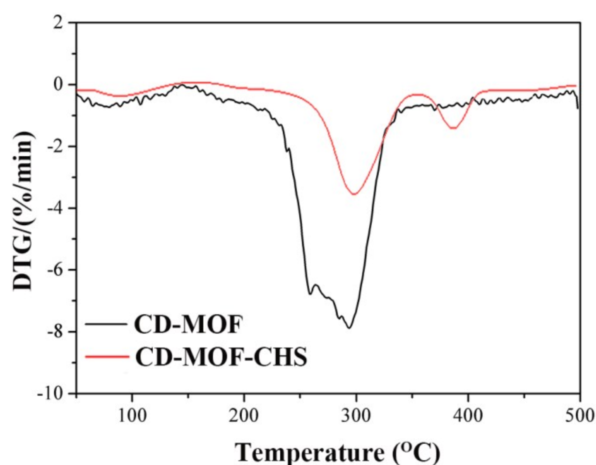

Figure S-3. Thermal gravimetric derivative (DTG) curves calculated from TG of CD-MOF and CD-MOF-CHS showing two step degradation of CHS modified CD-MOF (single step).

The weight loss of CD-MOF-CHS samples was recorded in two steps in comparison with single step degradation of CD-MOF. The complete DTG curves were also presented in Figure S-3. The second degradation step showed about loss might be represented the cholesterol linked CDs.

The elemental analysis of CD-MOF and CD-MOF-CHS was carried out using Scanning electron microscopy-Energy-dispersive X-ray spectroscopy (EDS) based elemental analysis technique (PHENOMWORLD, 800-07334). The uncoated samples were immobilized on a metal stub with double-sided adhesive tape then observed under

definite magnification using full BSD detector and 15kV power. Results are presented in table S-1.

Table S-1. The percentage ratio of key elements of CD-MOF and CD-MOF-CHS recoded by EDS analysis.

Element	CD-MOF (%)	CD-MOF-CHS (%)
Carbon	61.24±1.55	63.41±1.03
Oxygen	31.00±4.75	33.05±0.99
Potassium	9.07±1.98	3.39±0.74

HPLC method for drug adsorption study

An adsorptionstudy was carried out by dispersingCD-MOF-CHS (0.1 g) in an aqueous solution of doxorubicin hydrochloride (DOX) with a concentration of 500 mg/L(pH 7.0, 20 mL). The mixture was kept stirring at 100rpm at 25 °C. Next, 50µL of the suspension was withdrawn at a timeinterval of 5, 10, 20, 30, 40, 50 and 60 min and submitted to centrifugation to separate theCD-MOFcubes and recover the supernatants.The concentration of DOXin thesupernatants was determined byhigh performance liquid chromatography (HPLC) at the wavelength of 554 nm, which isthe characteristic absorption peak for DOX.Reversed phase separationwas performed on LunaTM analytical column Phenomenex C18(150*4.6mm,5µm) at room temperature. The mobile phasewas a mixture of methanol and 0.1% trifluoroacetic acid at a ratio of 50:50, and gradient elutionwas utilized, i.e., 1 mL/min until 14 min, linear increaseto 1.5 mL/min till 20 min, then maintainedfor 9 min (20-29 min) before returning back to 1 mL/min over 1 min. Detection was performed at λex/em 233 nm and injected volumes were 20µL.Calibration equations were obtained usingleast squares regression method on the nominalconcentration *versus* the peak height ratio of DOX to the internal standard. The results were fitted in pseudo order kinetics model for deep investigation of adsorption process by using following formula (1):

$$\frac{t}{qt} = \frac{1}{kqe^2} + \frac{t}{qe} \quad \text{.......} 1$$

where qt is the adsorption capacity at time t, qe is the equilibrium time, t is the time and q^2 is the rate constant of pseudo second order kinetics.

Solvation energy calculation method

The molecular structure of γ-CD was extracted from the single crystal structure of MOF-1 in literature[1]. This derivative structure of γ-CD was built using the Materials Visualizer module in Materials Studio (MS, Accelrys Inc.) 5.0. The Forcite module in MS was employed for performing energy minimization, molculculardynamicas (MD) and solvation energy calculation. In all the protocols, the γ-CD part of derivative γ-CD model was fixed. All the models were in vacuum. In the task of minimization, a smart algorithm was employed with the total energy of the system converged to less than 2.0 × 10-5 kcal·mol-1, the residual force to less than 0.001 kcal·mol-1·Å-1, the displacement of atoms to less than 1 × 10-5 Å. In the MD protocol, the COMPASS II forcefield was adopted, and the NVT ensemble and the Berendsen temperature control method were employed for a total period of time of 50 ps. The non-bond cutoff distance of 18.5 Å, spline width of 1.0 Å, and buffer width of 0.5 Å were used respectively. In the Solvation Free Energy protocol, the derivative γ-CD model was put in a box with the length of 50*50*50 $Å^3$. The derivative part was assigned as the solute atoms set. The thermodynamic integration algorithm was used for the free energy calculation. The other parameters were set as MD protocol.

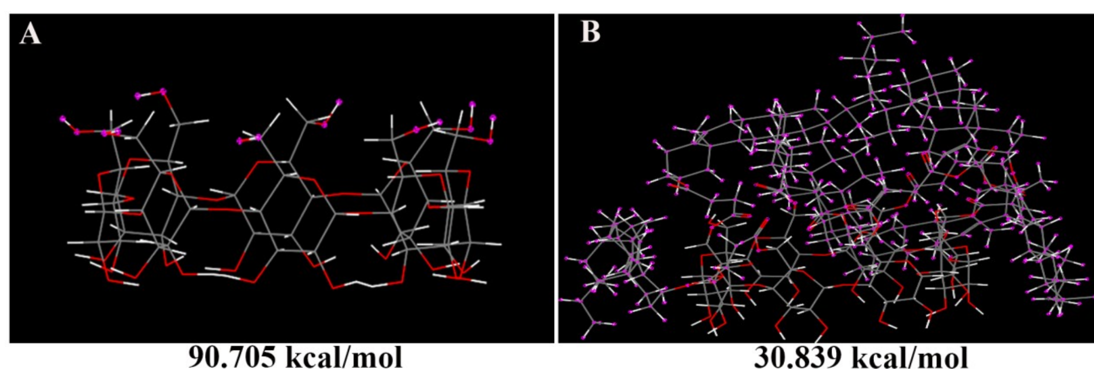

Figure S-4. Simulated structure of single CDand CHScoupled CDused for the calculation of solvation energy.

Sample preparation and HPLC method for cholesterol detection

The content of CHSin the CD-MOF-CHS samples was determined by HPLC(Agilent Technology, 1290 infinity) using an ELSD detector (model-Agilent Technology 1260

ELSD). The CHSwas recovered from the CD-MOF-CHS sample by hydrolysis of ester bonds using ammonia (NH_3). Briefly, 30 mg of CD-MOF-CHS samples was dispersed in 800 µL of Milli Q water and 200µL ammonia solution. Then, 2 mL of methanol was added and after 10 minutes sonication a clear solution was obtained.The samples were filtered through 0.22 µm membrane filters and injected into HPLC.

The CHS content in CD-MOFswas determined by reversephase separationchromatography method by using Unitary column C18,150*4.6mm,5µm) at 35°C. The isocratic mobile phase (methanol) was flowed for 10 minute with 1ml/min flow rate. The injection volume was set to 10µL.The samples peak was integrated by base to base method. Calibration equations were obtained usingleast squares regression method on the nominalconcentration *versus* the peak area ratio of CHSsuccinate to the internal standard.

The ratio x of CHS and CDs on the surface of CD-MOF was calculated using following mathematical equation (2):

$$[(6 * \left(\frac{200}{3.1006}\right)^2 * x * 486.78)/(\left(\frac{200}{3.1006}\right)^3 * 8406.62) = 0.032, x] \quad (2)$$

Description: The equation takes into account the 6 faces of CD-MOF crystals with a mean size of 200 nm. 3.1006 is the diameter of each MOF unit (nm). The molecular weight of cholesterol succinate and a unit of CD-MOFs, are 490.48 and 8406.62, respectively. The calculation was according to the 3.2% of CHS (wt%).

Human cell culture

HeLa cells were obtained from the Chinese Academy of Science Type Research Products (Shanghai, China) andcultivated in plates in DMEM supplemented with 10% (v/v) fetal bovine serum, 1% (w/v) L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were maintained at 37 °C in a 5% CO_2 water saturated atmosphere.

Table S-2. The percentage ratio of key elements of DOX loaded CD-MOF-CHS recoded by EDS

analysis.

Element	DOX-CD-MOF-CHS (%)
Carbon	54.94±2.31
Oxygen	38.25±1.92
Nitrogen	2.99±0.32
Potassium	2.79±0.22

Cellular uptake study

γ-CD-MOF particles were loaded with DOX(Figure S-5), a widely-used anticancer drug. The resulting particles were nearly amorphous,asshown by the absence of crystalline peaks in the XRD patterns (Figures S-6).

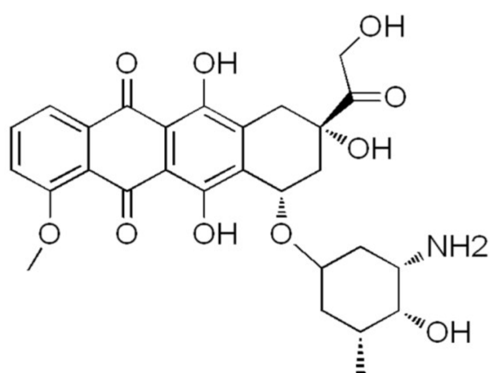

Figure S-5. Chemical structure of doxorubicin

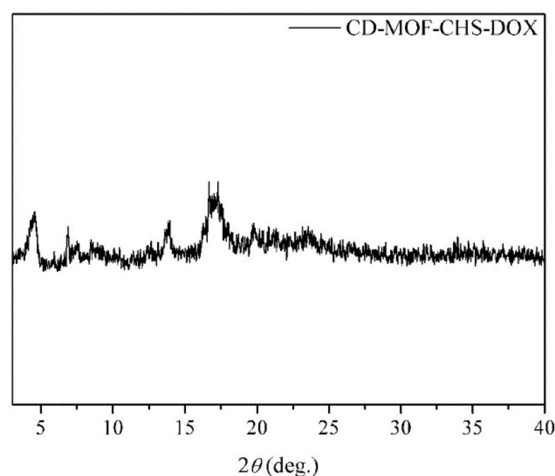

Figure S-6. PXRD pattern of CD-MOF-CHS after loading of doxorubicin

The cellular uptake of DOXand CD-MOF-CHSnanoparticles were visualized under confocal microscopy (Olympus FV 3000 confocal laser scanning microscope) and confocal images were processed by FV10-ASW-Viewer 4.0. Hela Cells were seeded

onto the glass bottom cell culture dish (Ø 15mm) at 2×10^4 cells/welland cultured overnight. Then, cells were respectivelyincubated with free DOXand CD-MOF-CHSparticles loaded with DOX at 37ºC for 4h. After that, the cells were labeled with DAPI to stain the lysosomes and nucleus.Theuptake of DOXin Hela cells was denoted as the red(525 nm) fluorescence signals.

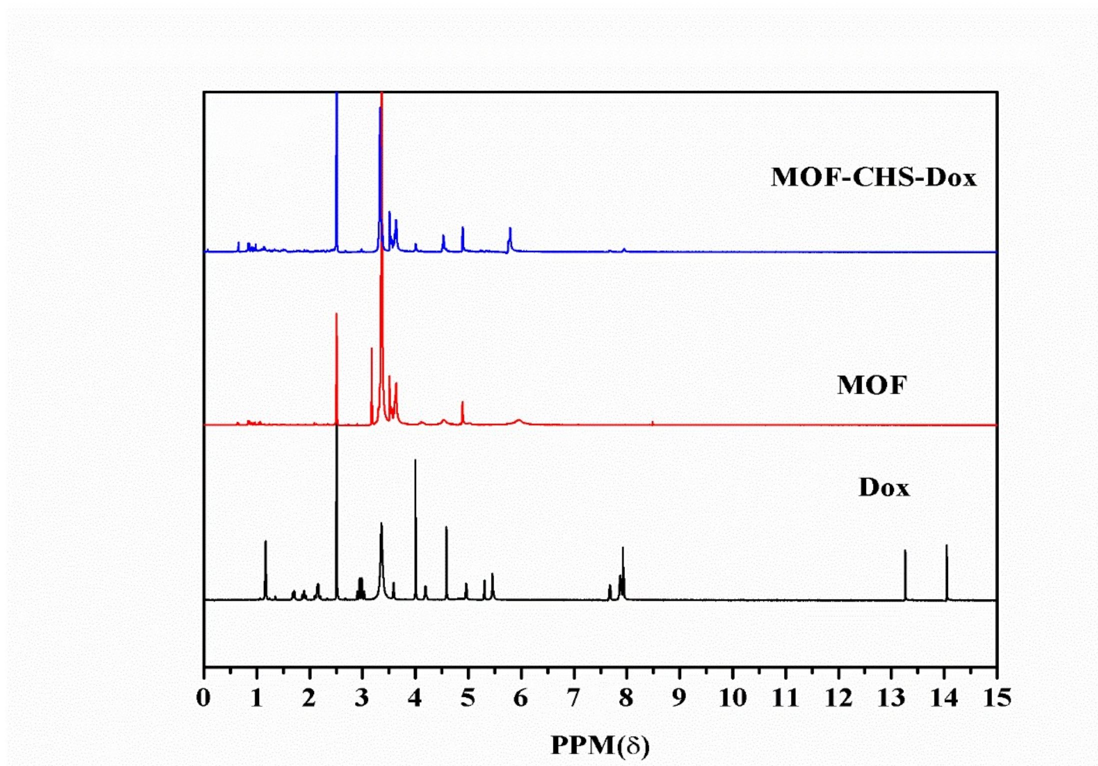

Figure S-7.1HNMR graph of DOX, CD-MOF-CHS and DOX loaded CD-MOF-CHS explaining the feature shift of DOX when loaded to CD-MOF-CHS structures.

Cell viability study

The *in vitro* toxicity of CD-MOF-CHS nanoparticleswas tested usingthe Enhanced Cell Counting Kit-8 (CCK-8, Beyotime). HeLa cells were seeded into 96-well plates and incubated for 24 hrs. Cells were treated with samples (20 µL) for 4 hrs. After that, CCK-8 solution (10 µL) was added to each well and incubated for 2 hrs. Each treatment was tested in six individual wells. At the end of the experiment, the absorbance was measured at 650 nm using a microplate reader (Thermo scientific, Multiscan Go). The cell viability was calculated by using the following formula (3):

$$\text{Cell Viability (\%)} = \frac{OD_{exp} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100 \quad (3)$$

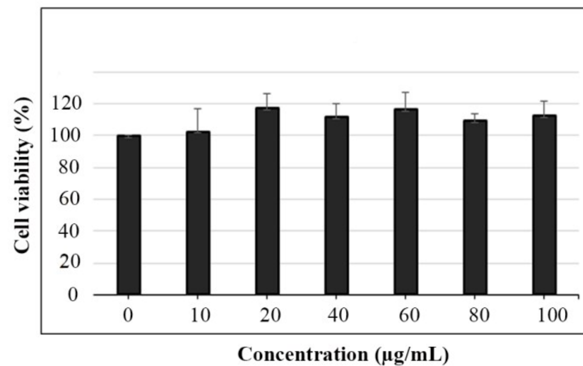

Figure S-8.MTT assay data show a biofriendly nature and no cytotoxic effects of CD-MOF-CHS nanoparticles on HeLa cells up to concentrations of 100 μg/mL.

Animal experiments

The 8-week male Sprague-Dawley rats weighting about 200 g and 6-week male BALB/c mice were purchased from Liao Ning Chang Sheng Biotechnology Co. Ltd. (Benxi, P. R. China) and Changchun Institute of Biological Products, Co. Ltd. (Changchun, P. R. China), respectively. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Animal Care and Use Committee of Jilin University.

***In vivo*pharmacokinetics study**

For the study of blood clearance of free DOX, and DOX loadedCD-MOF-CHSnanoparticles *in vivo*, male rats drank water only for 12 h before the test. Rats were divided in groups of 8 per each experiment. After intravenous administrations of free DOX, CD-MOF-CHS at an equivalent DOX dose of 5.0 mg kg per body weight (mg (kg BW)−1), 500.0 μL of blood was serially collected from the retrobulbar venous plexus into heparinized 1.5 mL Eppendorf (EP) tubes at 0.033, 0.083, 0.25, 0.5,1, 1.5, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120 and 144 h. To separate the plasma and blood cells, blood samples were centrifuged at 10,000 rpm and 4 °C for 10 min. 200.0 μL of supernatant was transferred into a new EP tube with 1.0 mL of methyl alcohol. After vortexing for 10 min, the samples were centrifuged again. The supernatants were

removed into glass tubes separately, and then the samples were blow-dried using a MD-12 nitrogen blowing instrument (Tapery, Nanjing, P. R. China) at 35 °C. Subsequently, the samples were redissolved in 200.0 μL of methanol, and the amount of DOX in various samples was detected by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A SCIEXMS system (USA) equipped with an Agilent 1100 HPLC system (USA) was used.The optimal MS parameters were as follows: The ESI source was operated in positive ionization mode; Scan Mode, MRM; Curtain gas, 15 psi; CAD gas, 3 psi; Ion source gas 1, 25 psi; Ion source gas 2, 45 psi; Ion-spray voltage, 5000V; temperature, 500℃; DOX m/z 544.3→397.1, internal standards (IS) m/z 528.3→321. All data were acquired by Analyst 1.5.1 (SCIEX, USA). DOX and IS were separated on a Agilent ZORBAX Extend-C18(150×4.6 mm, 5.0 μm) ,by gradient elution with the mobile phase of 0.1% formic acid aqueous (A) and 0.1% formic acid acetonitrile (B) at the flow rate of 1.0 mL/min. The gradient program was as follows: 0-1 min, 20% B; 1-3 min, 20-40% B; 3-4 min, 40-90% B; 4-5.5 min, 90%B; 5.5-6 min, 90-95% B, 6-6.1min, 95-20% B, 6.1-8min, 20% B. The column temperature was set at 40℃. An injection volume of 20 μL was usedfor all analysis. Results are presented in Table S-2.

Table S-3. Pharmacokinetics parameters including half-lives ($T_{1/2z}$), area under the curve ($AUC_{0\text{-}t}$), λ_z and mean residence time ($MRT_{0\text{-}t}$) for CD-MOF-CHS samples in comparison with free DOX intravenously administered at equivalent doses of 5 mg/kg in rats.

Parameters	Unit	Free Dox	CD-MOF-CHS
AUC(0-t)	ng/L*h	995.8±194.2	1550±112.9
AUMC(0-t)	h*h*ng/L	21389±3266	44435±11078
MRT(0-t)	h	21.82±3.203	28.45±5.269
λ_z	1/h	0.018±0.008	0.006±0.003
t1/2z	h	48.46±25.47	177.0±120.1

References

1. Singh, V.; Guo, T.; Wu, L.; Xu, J.; Liu, B.; Gref, R.; Zhang, J. RSC Adv. 2017, 7, 20789-20794.