Supplementary materials

Experiments

Materials

Chitosan was purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). NHS and EDC was purchased from shanghai Aladdin Company (Shanghai, China). Heparin sodium (Hep) was purchased from Macklin Biochemical Company (Shanghai, China). Doxorubicin (DOX) was purchased from Aladdin Company (Shanghai, China). Dimethylsulfoxide and formamide was purchased from Shanghai Jinshan Chemical Plant (Shanghai, China). All other chemicals were of analytical grade and used as received. Double-distilled water (18.2 MΩ) was obtained from Milli-Q system (Millipore, Bedford, MA, USA) and was used in the experiments. Adult whole blood was provided by Jiangsu blood centre. Dulbecco's modified eagle medium (DMEM) were purchased from Thermo Fisher HyClone (USA). Fetal bovine serum (FBS) was obtained from SunShine Biotechnology Co., Ltd. (China). Methyl thiazolyl tetrazolium (MTT) was purchased from Amresco (USA). Hoechst 33258 was purchased from Gibco Co., Ltd. (China). The mouse embryonic fibroblast (NIH3T3), human breast cancer (MCF-7), human cervical cancer cells (HeLa cells) and human pulmonary adenocarcinoma cell (A549 cells) were purchased from the Cell Bank of Culture Collection of Chinese Academy of Sciences (Shanghai, China).

Synthesis of functionalized CDs

Chitosan(0.5 g) was dissolved in 25 mL deionized water to form a homogeneous solution. After stirring for 30 min, the mixture was sealed into a 50 mL Teflon equipped stainless steel autoclave followed by hydrothermal treatment at 200 °C for 10 h. After cooling to room temperature, a black solution was obtained and named CDs. To further purify the CDs, the solution was centrifuged at 3000 rpm for 10 min to remove small volumes of liquid and filtered through a 0.22 μm membrane to remove large or agglomerated particles. The purification of the CDs was conducted through a dialysis tube (3500 Da molecular weight cut off) for about 24 h in dark [1]. The obtained CDs solution were dried to a powder by vacuum freeze-drying machine and stored in a refrigerator at 4 °C prior to use.

Synthesis of CDs-Hep

Firstly, the Hep (0.5 g) was evenly dispersed in 10 mL waterless formamide at N_2 condition,

then respectively added in 205 mg EDC and 115 mg NHS, and stirred for 12 h in a dark place. Secondly, the obtained coarsely colourless solution was filtered by a 0.22 μm syringe filte to remove the black residue. Thirdly, the solution was precipitated by acetone. Finally, a light white sticky solid was obtained and kept under vacuum at 60 °C for 24 h.

The Hep-NHS (0.3 g) was dispersed in waterless formamide (5 mL) and waterless dimethylsulfoxide (5 mL) at N2 condition, then respectively added in 50 mg CDs and triethylamine (1%), and stirred for 12 h in a dark place. After being concentrated, the solution was precipitated by acetone. To further purify the CDs-Hep, the solid was washed with ethanol to remove residual chemicals, separated from unbound Hep. Finally, the obtained CDs-Hep solution were dried to a powder by vacuum freeze-drying machine and stored in a refrigerator at -20 °C prior to use. The quality of the Hep was calculated by azure A colorimetric method.

The Hep content =(weight of drug in nanoparticles)/weight of nanoparticles)× 100%

Instruments and Characterization

The photoluminescent (PL) spectra were recorded using molecular fluorescence spectrometer (Cary Eclipse, varian, USA). (FT-IR) Nexus 670 FTIR type (Nicolet) infrared spectrometer was used to analyze the infrared spectrum of the sample. The test conditions were: resolution 4 cm-1, scanning times 64 times, scanning range 4000~500 cm-1, potassium bromide tablet. The fluorescence lifetime were recorded using the Time-resolved Fluorescence Life Time Microscope (FM-4P-TCSPC, Horiba Jobin Yvon, USA). The X-ray diffraction (XRD) analysis was performed using a D/Max 2500V/PC diffractometer (Rigaku Corporation, Japan). The test conditions were: Cu target, K alpha ray, tube voltage 40 kV, tube current 100 mA, scanning speed of 2 degrees/min. The thermogravimetric measurements were performed on a Perkin-Elmer TG 7 instrument using a heating rate of 10 °C/min up to 800 °C in nitrogen atmosphere. Ultraviolet-visible (UV-Vis) absorption spectra were recorded using UV absorption spectrophotometer (Cary-50, varian, USA). Proton nuclear magnetic resonance spectroscopy (1H NMR) was tested by 400 MHz WB Solid-State NMR Spectrometer, Switzerland. The surface composition and element analysis of the samples were recorded using X-ray photoelectron spectroscopy (XPS, EscaLab-250, Thermo, USA). The Malvern ZEN 3600 Zetasizer (Malvern Insruments, UK) was used to study the prepared hydrodynamic size and Zeta potential. The morphologies of the samples were characterized using a transmission electron microscope (TEM, H-7650, Hitachi, Japan). The

fluorescent images of cells were acquired by laser scanning confocal microscope (TI-E-A1R, Nikon, Japan) and upright fluorescent microscope (XSP-63X, Nikon, Japan).

Quantum yield (Φ) measurements

Specific experimental procedure is as follows: firstly, a small amount of the quinine sulfate (Φ_{st} = 0.54) was dissolved in 0.1 M solution of H_2SO_4, and the CDs were dissolved in deionized water. Then, the absorbance values of CDs and quinine sulfate were measured at 360 nm. Ensuring both of the absorbance values were less than 0.1. In addition, the integral fluorescence intensity of CDs and quinine sulfate were obtained by measuring both of PL emission spectra at an excitation wavelength of 360 nm. Finally, the quantum yield of CDs was calculated using following equation:

$$\Phi = \Phi_{st} (K_x / K_{st}) (\eta_x / \eta_{st})^2$$

Where Φ is the quantum yield, K is the slope determined by the curve, and $\eta_x / \eta_{st} = 1$. The subscript "st" and "x" denote the the standard and samples, respectively.

Drug loading

DOX was loaded into the CDs-Hep by dialysis method as described in previous studies. To prepare the blank CDs-Hep solution, CDs-Hep (0.2 mM) were dissolved in ultrapure water followed by treatment of ultrasonicator at room temperature. DOX (0.1 mg/mL) was added into the yellow CDs-Hep solution and stirred for 12 h in a dark place [2]. Following the mixed solution was subject to dialysis (molecular weight cutoff of 1000 Da) against deionized water for 48 h. The quantitation of DOX calculated by the absorbance of DOX at 485 nm.

DOX loading ratio=(weight of drug in nanocarrier/weight of nanocarrier taken) × 100%

Size stability test

In the preparation of CDs-Hep nanoparticles, the quality of Hep-NHS was divided into four groups: 200, 300, 400 and 500 mg. The CDs-Hep nanoparticles dissolved in 3 mL ultrapure water and then the size of CDs-Hep was measured by dynamic light scattering (DLS) measurements. The morphologies of the samples were characterized by transmission electron microscope (TEM). The freeze-dried CDs-Hep/DOX (10^{-4} g/mL) dissolved in deionized water, then adjusted the dispersions of pH and temperature, and used dynamic light scattering (DLS) instrument to measure CDs-Hep/DOX diameter at different pH and temperature.

In vitro drug release study

The drug release test was investigated in a phosphate buffer saline (PBS) medium at pH 7.4, 6.5 and 5. The CDs-Hep/DOX (10 mL) was dialyzed in buffer solution at pH 7, 6.5 and 5 and kept in the dark and stirred slowly. Finally, the concentration of dissociated DOX and the cumulative release rate with respect to the buffer solutions was monitored based on the absorbance of DOX at 488 nm [3]. The amount of released Hep was calculated by azure A colorimetric method. The amount of Hep from CDs-Hep/DOX was calculated according to the standard curve of Hep.

Cell imaging

To study mammalian cell imaging, the cellular uptake of DOX and CDs-Hep/DOX by A549 cells was investigated using a confocal laser scanning microscope (CLSM). The cells were seed at a density of 1×10^5 cells/well onto 35-mm Petri dish and incubated at 37 °C for 12 h. Then DOX and CDs-Hep/DOX solution was diluted to a final concentration of 300 μg/mL by liquid medium and then cell were incubated with DOX and CDs-Hep/DOX for 3 h at 37 °C. The cells monolayer was washed three times with phosphate-buffered saline (PBS, PH=7.4) and fixed on a solid with 3% paraformaldehyde for 30 min [4]. Cellular uptake and DOX delivery were detected using a confocal laser scanning microscope.

whole blood adhesion test

The DOX, CDs, Hep, CDs-Hep and CDs-Hep/DOX (0.005%) were dispersed in PVA, and then mixed into a glass surface dish, and placed at room temperature for 72 h. Then, the samples were cut into square pieces and then immersed in PBS for 24 h, and transferred to 24-well microtiter plate. After that, added 0.5 mL whole blood, and the 24-well micro titer plate was kept at 37 °C. The samples were washed by PBS 3 times, and the platelet were fixed by glutaraldehyde solution (2.5%, V/V) for 30 min [5]. Finally, samples were dehydrated by different ethanol (25%, 50%, 70%, 80%, 90%, 100%) solutions respectively. The whole blood adhesion surfaces were coated with gold prior to being observed by SEM (JSM Model 6300 Scanning electronic microscopy, JOEL, Japan).

Cell viability assay

We assessed vitro cytotoxicity of CDs, DOX and CDs-Hep/DOX by the MTT assay in NIH3T3, MCF-7, A549 and HeLa cells. At first, the NIH3T3, MCF-7, A549 and HeLa cells were cultivated in culture flask which containing DMEM (High Glucose) replenished with 10% fetal bovine serum, 1% penicillin and streptomycin mixed solution at 37 °C filling with 5% of

CO_2. The NIH3T3, MCF-7, A549 and HeLa cells were lifted with 1 mL trypsin-EDTA when cells covered approximately 80% confluence in the flask. Then, the NIH3T3, MCF-7, A549 and HeLa cells were seeded into 96-well culture plates with 100 μL cells suspension. After overnight culture, discarding the original DMEM of 96-well culture plates, the solutions of CDs, DOX and CDs-Hep/DOX with concentrations at 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05mg/mL were added to each wells, respectively, each of which was repeated in triplicate. After the NIH3T3, A549 and HeLa cells were incubated at 37 °C for 24 h and 48h, respectively, then each well were added to 20 μL MTT and incubated for an additional 4 h, the medium of each wells was depleted and 100 μL dimethyl sulfoxide (DMSO) was added to each wells to generate bluish violet formazan [6]. Finally, We immediately measured the optical density (OD) of each well at 570 nm on BioTek synergy 2 Multi-Mode Microplate Reader (BioTek, USA) to assess the cell viability.

$$\text{Cell inhibition rate} = [1-(D_t/D_b)] \times 100\%$$

where D_t is the absorbance of sample; D_b is the absorbance of blank control group.

Activated partial thromboplastin time (APTT) test

Firstly, the Hep standard solutions with concentrations at 0.15 U/mL, 0.3 U/mL, 0.45 U/mL, 0.6 U/mL and 0.75 U/mL was prepared. Secondly, the CDs-Hep and CDs-Hep/DOX (3 μg/mL) were dissolved in ultrapure water followed by treatment of ultrasonicator at room temperature. Similarly, we selected the slow-release fluid of CDs-Hep and CDs-Hep/DOX as experimental group. Thirdly, Hep standard solution or sample solution (25 μL) was added in 0.1 mL plasma, then the mixture was added in APTT (0.1 mL) solution. The mixture was placed in a 37 °C incubator, and the preheated $CaCl_2$ (0.1 mL) solution was added [7]. Finally, the coagulation time was recorded by PRECIL C2000 coagulation instrument.

Hemolytic test

The hemolytic test was tested to determine blood compatibility of CDs, DOX, CDs-Hep and CDs-Hep/DOX. Fresh anticoagulated blood from human volunteers (2 mL) was diluted with 2.5 mL of normal saline solution. The CDs, DOX, CDs-Hep and CDs-Hep/DOX (0.01 mg/mL) were prepared in normal saline solution and kept at 37 °C for 30 min. The diluted blood (0.2 mL) was added in CDs, DOX, CDs-Hep and CDs-Hep/DOX. The mixture was kept at 37 °C for 60 min, and then centrifuged at 1500 rpm for 10 min. The supernatant was transferred to a 96-well plate where the absorbance was measured at 545 nm using a BioTek synergy 2 Multi-Mode Microplate

Reader. Positive controls consisted 0.2 mL diluted blood in 10 mL deionized water while negative controls consisted 0.2 mL diluted blood in 10 mL normal saline solution [8]. Hemolysis degree was calculated as follows:

$$\text{Hemolysis rate} = [(D_t - D_{nc}) / (D_{pc} - D_{nc})] \times 100\%$$

where D_t is the absorbance of sample; D_{nc} is the absorbance of the negative control; and D_{pc} is the absorbance of the positive control.

Platelet adhesion test

The DOX, CDs, Hep, CDs-Hep and CDs-Hep/DOX (0.005%) were dispersed in PVA, and then mixed into a glass surface dish, and placed at room temperature for 72 h. Then, the samples were cut into square pieces and then immersed in PBS for 24 h, and transferred to 24-well microtiter plate. After that, added 0.5 mL platelet rich plasma (PRP), and the 24-well micro titer plate was kept at 37 °C. The samples were washed by PBS 3 times, and the platelet were fixed by glutaraldehyde solution (2.5%, V/V) for 30 min [4]. Finally, samples were dehydrated by different ethanol (25%, 50%, 70%, 80%, 90%, 100%) solutions respectively.

References

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Figure Caption

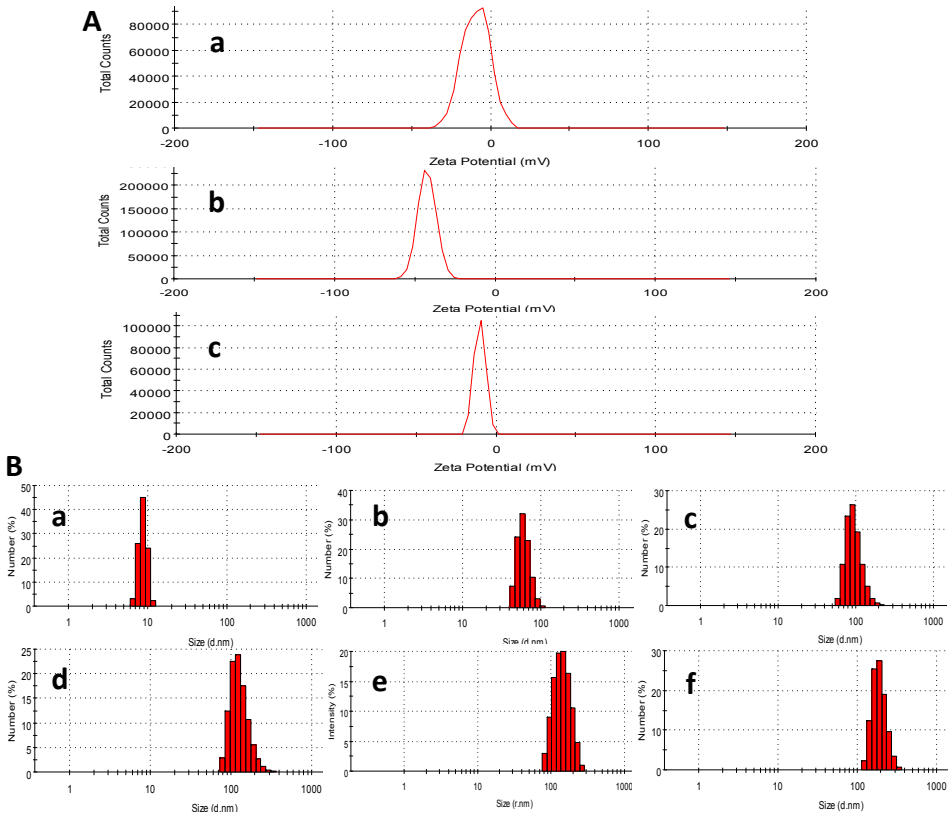

Fig. S1 (A) Zeta potentials of (a) CDs, (b) CDs-Hep and (c) CDs-Hep/DOX. (B) The size distribution of (a) CDs, (b) CDs-Hep (contain Hep-NHS 200 mg), (c) CDs-Hep (contain Hep-NHS 300 mg), (d) CDs-Hep (contain Hep-NHS 400 mg), (e) CDs-Hep (contain Hep-NHS 500 mg) and (f) CDs-Hep/DOX.

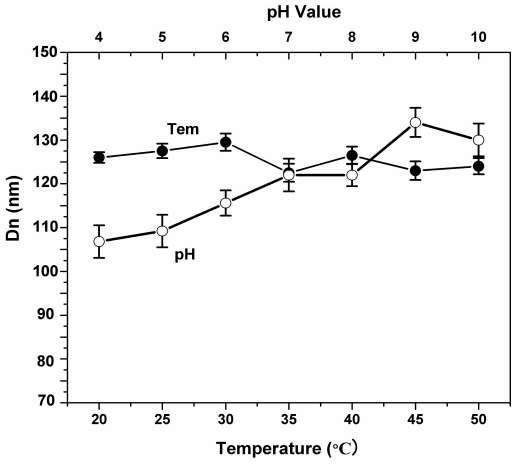

Fig. S2 pH and temperature dependence of the mean hydrodynamic diameter of the CDs-Hep.

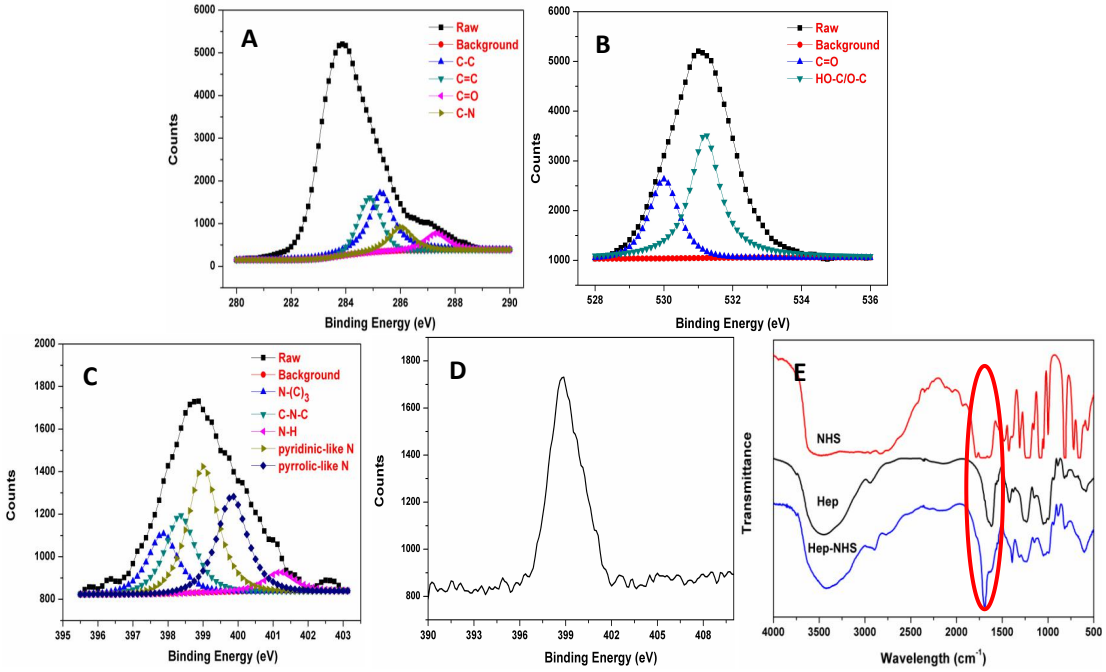

Fig. S3 (A) C 1s, (B) O 1s, (C) N 1s, and (D) N1s signals of CDs. (E) FT-IR spectra of NHS, Hep and Hep-NHS.

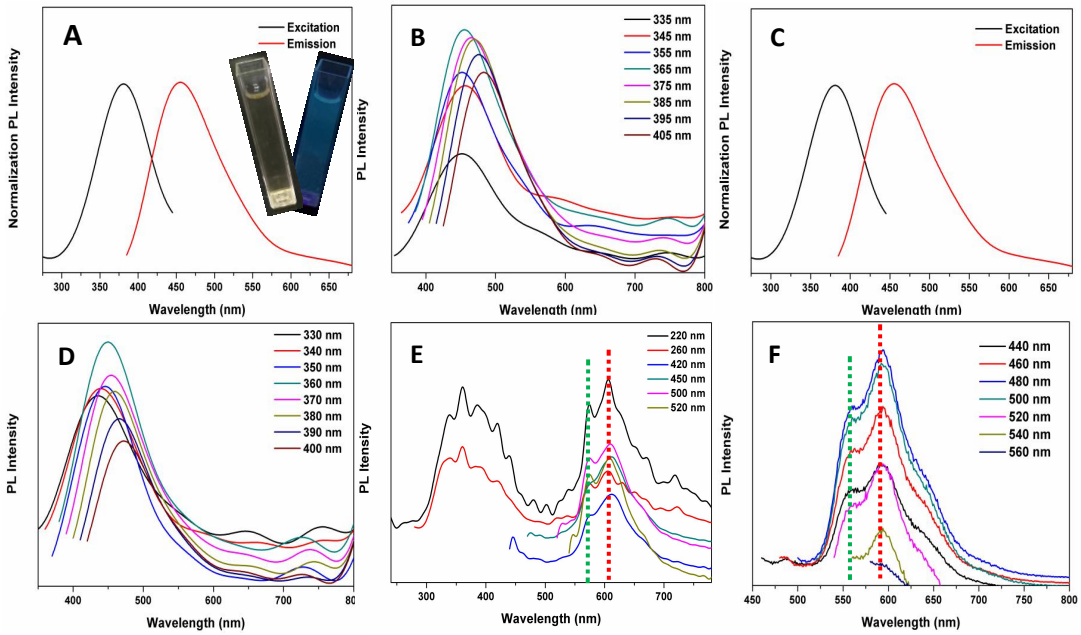

Fig. S4 (A) Fluorescence excitation and emission spectra of CDs. (B) Excitation-dependent emission shift of CDs-Hep. (C) Fluorescence excitation and emission spectra of CDs. (D) Excitation-dependent emission shift of CDs-Hep. (E) Excitation-dependent emission shift of CDs-Hep/DOX. (F) Excitation-dependent emission shift of DOX.

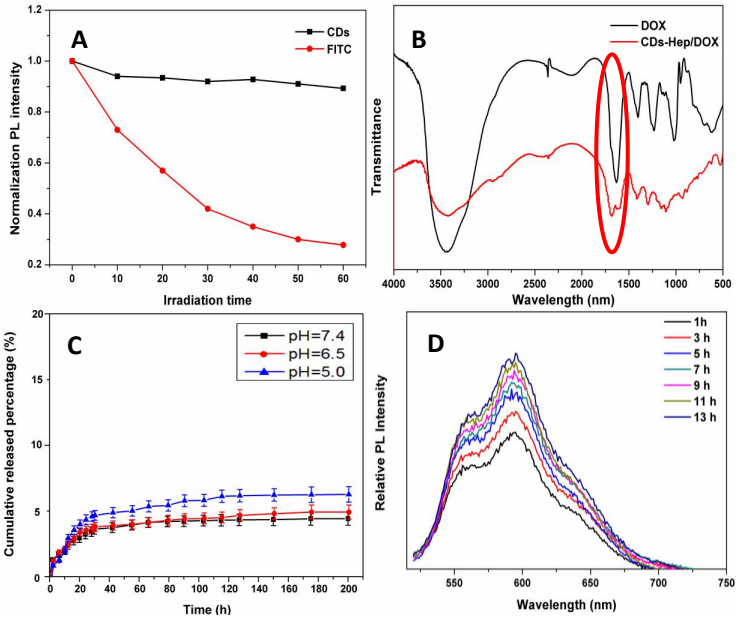

Fig. S5 (A) Comparison on the photobleaching characteristics of CDs and FITC (a 500 W xenon lamp). (B) FT-IR spectra of DOX and CDs-Hep/DOX. The release profile of DOX. (C) Time-dependent fluorescence spectra of drug release after incubating CDs-Hep/DOX at pH 6.5. (D) The release profile of Hep from CDs-Hep/DOX system in different buffer.

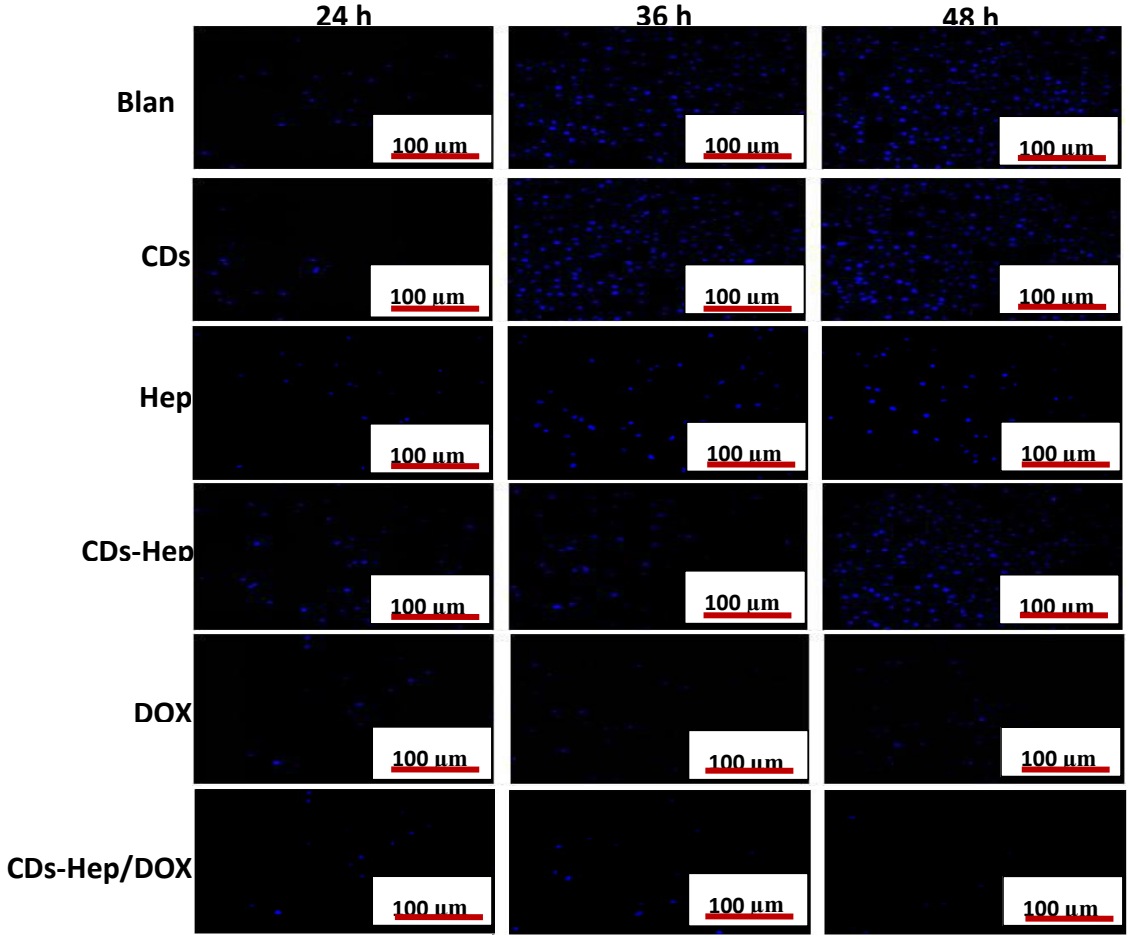

Fig. S6 HeLa cells stained with Hoechst 33342 after incubating in various media.

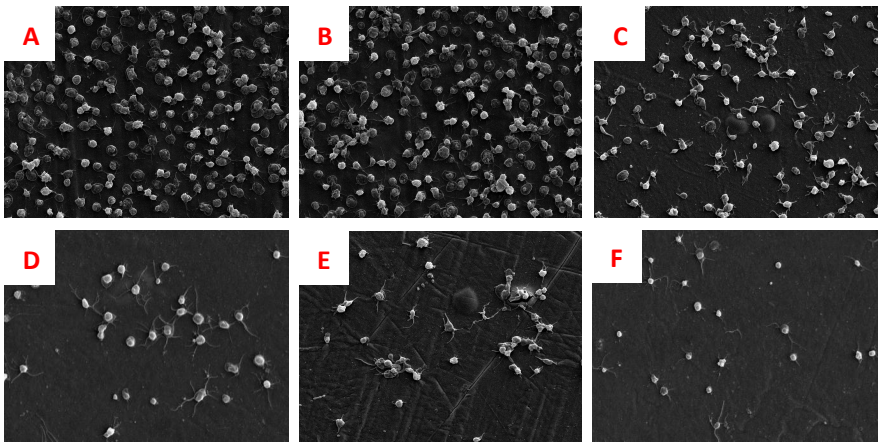

Fig. S7 Platelet adhesion of blank control (A), DOX (B), CDs (C), Hep (D), CDs-Hep (E) and CDs-Hep/DOX (F).

Fig. S8 Special handling images of Fig. 6 (A). (The CDs and CDs-Hep enter spider plant and be distributed to different tissue parts were observed in special handling images).