Supporting Information (SI) for

A tumor microenvironment model coupled with mass spectrometry to probe the metabolism of drug-loaded nanoparticles

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Supporting Information includes experimental section (materials, cellular uptake, cell analysis and chip-mass spectrometry for paclitaxel detection); fabrication of microfluidic Device (Figure S1); evaluation of the mass diffusion in the co-culture microfluidic channels (Figure S2); cell co-culture on vessel network microchip (Figure S3, 4); on-chip generation of rigid paclitaxel-laded nanoparticles (Figure S5, 6); characterization of rigid Paclitaxel-laded nanoparticles (Figure S7, 8); and detection of paclitaxel metabolites by microvessel-ESI-MS (Figure S9).

Experimental Section

Materials.

Silicon wafers were obtained from Xilika Crystal polishing Material Co., Ltd. (Tianjin, China). Negative photoresist (SU-8) and the developer were purchased from Microchem Corp. (Newton, MA, USA). Poly (dimethylsiloxane) (PDMS) and the curing agent were obtained from Dow Corning (Midland, MI, USA). Coverslip was bought from Beijing Biodee Biotechnology Co. Ltd. A human cervical carcinoma cell line (Hela) and human umbilical vein endothelial cells (HUVECs) were purchased from Cancer Institute and Hospital (Chinese Academy of Medical Science, Beijing, China). Dulbecco modified Eagle's minima essential medium (DMEM), fetal bovine serum (FBS), phosphate buffer saline (PBS) and 0.25% trypsin with EDTA were from Gibco, Grand Island, NY. Dimethylsulfoxide (DMSO) was purchased from sigma. The packaging material for the cell secretion pretreatment was obtained from the SPE C18 (macropore) column (Agela, Tianjin, China). A live/dead assay kit (Calcein-AM/EthD-1) was obtained from Invitrogen. Dihydroethidium dyes were purchased from Beyotime. 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, 2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000 (DSPEPEG2000) were purchased from Avanti (USA). Poly (D, L-lactide-coglycolide) (PLGA, lactide: glycolide = 75:25) was purchased from SurModics (USA). Dimethylformamide (DMF), trifluoroethanol (TFE), 2,3-Naphthalenedicarboxaldehyde and Paclitaxel (99%) were purchased from Sigma. All reagents were of analytical reagent grade and were used without further purification. A syringe pump (KDS100, kdScientific, Holliston, MA, USA) was employed to deliver eluting solutions in accurate rates. A plasma cleaner (PDC-32G, Harrick Plasma, Ithaca, NY, USA) was used for oxygen plasma treatment. A fluorescence microscope (Leica DMI 4000 B, Wetzlar, Germany) equipped with a CCD camera was used to observe and to obtain images of the microfluidic devices. The dimensions of each microstructure were manually measured by using Leica Application Suite, LAS V2.7. The stereomicroscope (XSZ-G, COIC, Chongqing, China) with the extended light

source (Leica CLS 100X, Leica, Wetzlar, Germany) was used for alignment of microfluidic device. ESI-Q-TOF detection was performed with a Bruker microTOF-Q mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). All mass spectra were obtained in the positive mode. MS image analysis was performed with DataAnalysis (Bruker Daltonics Inc.). A 500 μL syringe was obtained from Hamilton (Bonaduz AG, Switzerland).

Cellular uptake.

For cellular uptake analysis, Hela cells and HUVECs cells were co-cultured in microfluidic device and grown for 24 h for attachment. The cell culture medium was then changed to fresh DMEM medium without FBS but containing 5μg/ml rigid paclitaxel-laded nanoparticles and injected into the two HUVEC cell culture chambers to replace original cell culture medium. After rigid paclitaxel-laded nanoparticles were diffused into the Hela cells chamber under static conditions, the inlet of chamber containing HUVECs was connected to a syringe pump via poly (tetrafluoroethylene) (PTFE) tubes for supplying medium continuously at a flow rate of 5 μL/h. The medium could flow into the chamber containing Hela cells through the connection channels and flow out from the outlet at the Hela cell chamber. For cell culturing, the microchip device was put into a Petri dish and placed in the cell incubator with 5% CO_2 at 37 °C for analysis of the cellular uptake for each 12 h, 24 h, 36h, 48 h, 72 h. Fluorescence images were captured by laser scanning confocal fluorescence microscopy (Carl Zeiss LSM780, Carl Zeiss, Germany) and the data were analyzed by the program Image J software (NIH, version 1.49). All the tests were carried out in triplicate to give the average values.

Cell proliferation, viability, apoptosis, and analysis of reactive oxygen species and glutathione levels.

Cell proliferation was monitored by CCK-8, and the data were collected by microplate reader. Cell viability was determined by calcein-AM/EthD-1 staining (live/dead kit). Live/dead solution was prepared by diluting 0.5 μL of calcein AM and

2.0 μL of ethidium homodimer into 1 mL of PBS. Incubation was carried out at 37 °C for 30 min after staining with the live/dead kit. Cell culture channels were then rinsed with PBS three times. After washing, fluorescence images were taken by CCD camera on a fluorescence microscope. For determination of cell apoptosis, ROS or GSH levels, cells were incubated with 100μM Hoechst 33342, 100 μM DHE (λex = 535 nm, λem = 610 nm), or 200 μM 2,3-naphthalene dicarboxaldehyde (NDA, λex = 460 nm, λem = 530 nm) at 37 °C for 30 min, followed by washing with PBS three times, before observation under the fluorescence microscope.

Chip-Mass Spectrometry for Paclitaxel Detection.

The paclitaxel metabolism of nanoparticles was studied at various time and various concentration intervals of cell medium by ESI-Q-TOF-MS (Bruker Daltonics Inc., Billerica, MA). The microfluidic chip was combined with ESI ion source by a fused-silica capillary (i.d., 50 μm; o.d., 365 μm) with poly (tetrafluoroethylene) (PTFE) cannula. The microfluidic vascular network were connected to micro-SPE column by PE tubes with an inner diameter of 1.4 mm and an outer diameter of 1.6 mm. The whole chip−ESI-MS system, therefore, can be designed as a quantitative analysis platform with sensitivity, selectivity, and high-throughput assay. In the pre-treatment section, the micro-SPE column is 80 μm high and 1.8 mm wide. The micro-SPE columns were preconditioned by washing with methanol followed by water. After loading of cell medium containing paclitaxel, the micro-SPE columns packed with C-18 particles were washed by 5% methanol and then eluted by 5% ammoniated methanol (v/v) solution for direct injection into the mass spectrometer at a flow rate of 3.0 μL min^{-1}. Full scan spectra within an m/z range from 50 to 1500 were calibrated by ESI tune mix. The MS analysis was operated in the positive-ion mode. The end-plate offset was −500 V, with the inlet capillary voltage at −4.5 kV. The flow rate of dry gas was 9.0 L/min, and dry temperature was 180 °C. A coaxial nebulizer N_2 gas flow around the ESI emitter was used to assist the generation of ion. MS analysis was carried out using argon as a collision gas to split precursor ions via collision-induced dissociation prior to mass analysis.

Fabrication of microfluidic device.

In order to investigate the drug delivery of nanoparticles from vasculature to tumor, the microfluidic cell co-culture device was designed to simulate an abnormal tumour vessel network system for drug evaluation. Based on the previous work,[1-2] we simulated the perivascular tumor microenvironment more realistically. This highly heterogeneous vascular network microfluidic device was developed to recapitulate the key features of complex transport of drugs and NPs within the tumor microenvironment. The microfluidic devices were fabricated by standard soft lithography and replica molding techniques.[2] The model was divided into three zones, the two external HUVEC cells culture chanels (60 μm high, 1.5 mm wide, and 13 mm long for each channel) representing blood vessels that could deliver the cell culture medium and drug solutions, the one internal Hela cells culture chamber (60 μm high, 2.0 mm in diameter) (Figure S1). These dimensions allowed a smooth loading and sedimentation of the cells. The two symmetrically distributed microvessel network channels connected the external and internal cell culture chambers (10 μm high), in order to prevent a hybridization of loaded cells from different inlets. This highly heterogeneous vascular network architecture had different widths (10, 20, 30 μm wide) which varied in channel flow pathway lengths. Furthermore, the connection was large enough to enable a diffusion of chemicals between the neighbor channels. To fabricate the microchannels, negative photoresist SU-8 2050 was spun onto a silica wafer at a speed of 2500 rpm for 50.0 s by a spin-coater. After baking at 65.0 °C for 10 min, UV light exposure and SU-8 developing were performed to produce the layer of connection channels (10 μm high). The wafer was loaded for 30 min before the second exposure to avoid an uneven surface. Another layer of SU-8 2050 photoresist was then coated onto the same wafer at a speed of 1800 rpm for 50.0 s. After baking at 65 °C for 10.0 min, UV light exposure and SU-8 developing were carried out to produce the layer of cell culture chambers and main channels (60 μm high). In order to fabricate the micro-SPE channel, we used SU-8 2007 at a spin speed of 1300 rpm to get a height of about 10 μm, and then the height was increased to about 80 μm using SU-8 2050 at a spin speed of 1800 rpm. After completion of these parts, salivation reagents and the silica master were put into

vacuum kettle together to make the master hydrophobic. Premixed poly-(dimethylsiloxane) (PDMS) prepolymer and curing agent were poured onto the mold and degassed under vacuum for 30 min. After curing at 80 °C for 2 h in oven, the PDMS was peeled off and cut into the designed shape. The prepared PDMS layers were sealed with a glass slide irreversibly after oxygen plasma treatment for 90 s. The cell co-culture micro-channels were modified with poly-L-lysine for a better cell adherence.

Figure S1 Microfluidic device for cell co-culture, metabolite analysis and cytotoxicity assay. (a) An image of the microfluidic device filled with green and red dye in the Hela cell culture and Huvec cell culture part, (b) Microscopic images of the spatial microstructure of the vessel network chip model.

Evaluation of the mass diffusion in the co-culture microfluidic channels.

In order to examine solute exchange between two main channels through the connection channels, we first perfused water-soluble blue dye into two side channels, followed by staining for dye diffusion (Figure S2). As expected, the blue dye efficiently diffused into connection channels within 5 to 15 min. A water-soluble blue dye balance between three channels could be observed in 45 min after the injection of the water-soluble blue dye solution. This observation indicates that the cell co-culture component allows efficient factor signaling between Hela cells and HUVECs during cell co-culture.

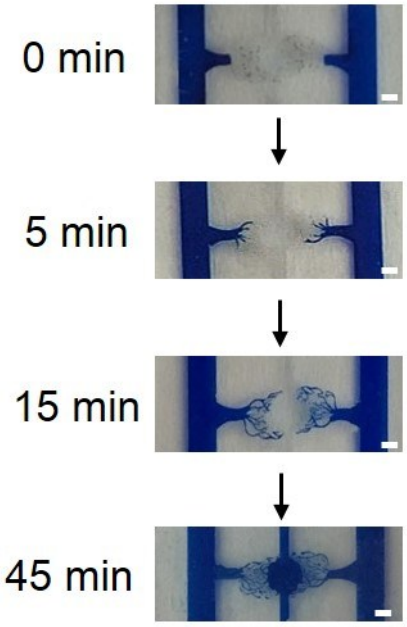

Figure S2 Evaluation of the mass diffusion in the co-culture microfluidic channels. Images obtained from 0 to 45 min after blue dye was injected into the both side channels. Scale bar: 1.0 mm.

Cell co-culture on vessel network microchip.

Human cervical carcinoma cell line (Hela) or HUVECs were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 units/mL streptomycin inside the cell incubator with 5% CO_2 at 37 °C for 2-3 days to ensure the cells in the exponential growth phase prior to microfluidic experiments. Two kinds of cells were then trypsinized and removed from the Petri dishes, followed by centrifugation and resuspension to a density of 10^7/mL in cell culture medium. FBS was injected and remained for 30 min to modify the glass slide surface and then washed by PBS for 3 times. Before cell seeding, the modified cell culture chamber was sterilized under UV light for 30 min. Then, 10 μl HUVEC cell suspension was added into the two external cell culture chambers respectively, after HUVEC cells settling, 5 μl Hela cell suspension was added into internal cell culture chambers. The cell suspensions would not enter into the transport channel due to the surface tension plug at the height difference junction of the channels and chambers. The device was placed into incubator for 2h allowing cell attachment to the substrate and then to be covered with a thin medium layer on the top surface of the microchip to avoid liquid evaporation. After 12 hours of continuous cell growth in the incubator, medium was filled into the inlets of two HUVEC chambers with their outlets plugged with solid PE columns to achieve liquid communication among different chambers. Subsequently, the cells were co-cultured with the medium changed every 12 hours. The viability of two types of cells was evaluated by a Live/Dead assay kit for three days and the data were analysed by Image J 8.0.

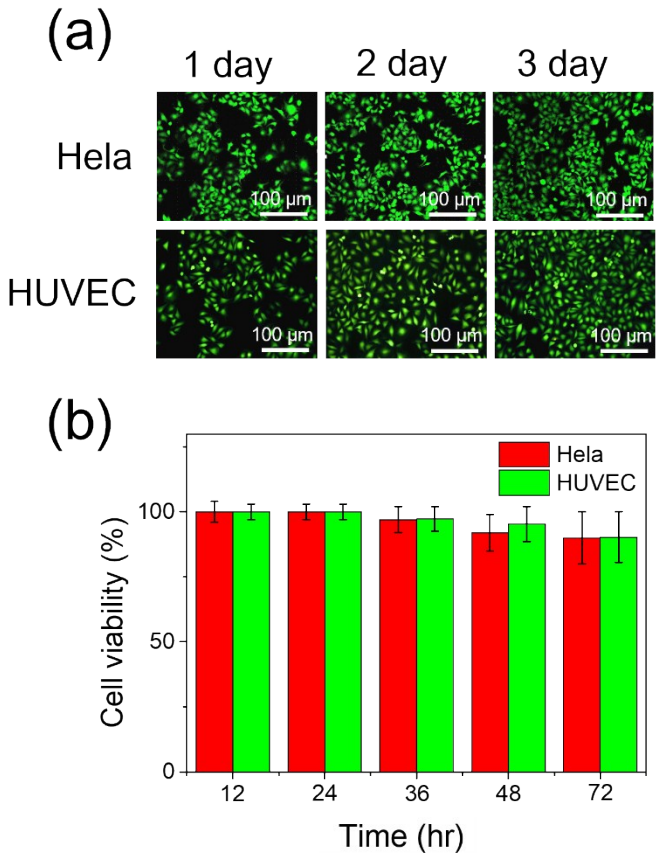

Figure S3 Viability of co-cultured Hela cells and HUVECs within the cell co-culture component for 72hr. (a) Fluorescent images of both cells lines by fluorescence microscopy. Calcein-AM/EthD-1 is used for cell staining. (b) Viability of Hela cells and HUVECs co-cultured in different main channels. Experiments are repeated three times in parallel.

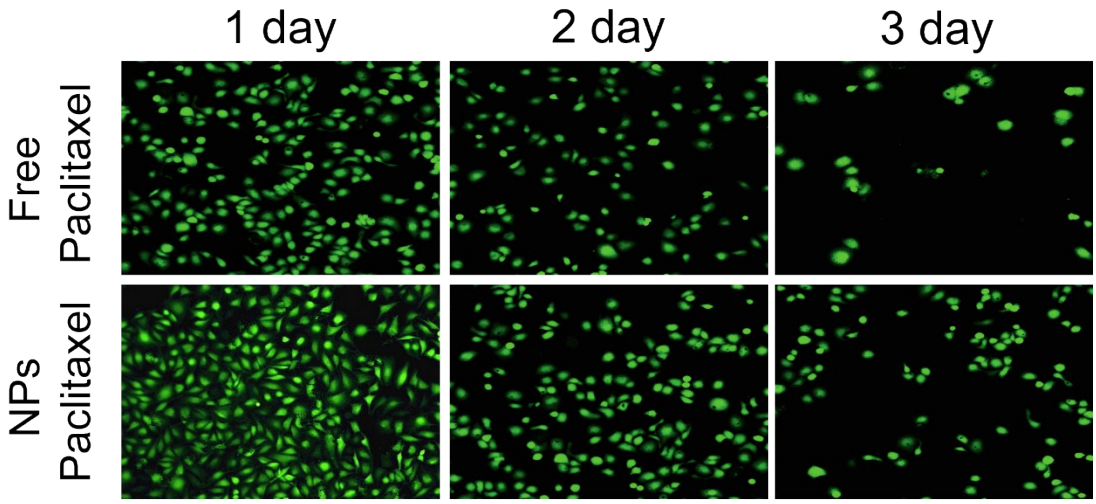

Figure S4 Fluorescent images of cocultured Hela cells treated with NPs paclitaxel and free paclitaxel (5.00 ng/mL) for three days and stained with calcein-AM. Green fluorescence indicates live cells.

On-chip generation of rigid paclitaxel-laden nanoparticles.

The microfluidic device for generation of rigid paclitaxel-laded nanoparticles consisted of two stages with four inlets and one outlet (Figure S5). The design and fabrication details are elaborated in the Supporting Information: (1) the first stage is composed of three inlet micro-channels connected to a straight microchannel for precipitation of a polymer core of hybrid nanoparticles; (2) the second stage is composed of one middle inlet and a double spiral micro-channel for self-assembling of lipid shell onto polymer core to form hybrid nanoparticles, which are collected from the outlet. The inlet channels in the first stage are 100 μm in width and 50 μm in depth. The other microchannels are 300 μm in width and 50 μm in depth. More details of microfluidic chip design can be found in the previous publication.[3-6] (Figure S6).

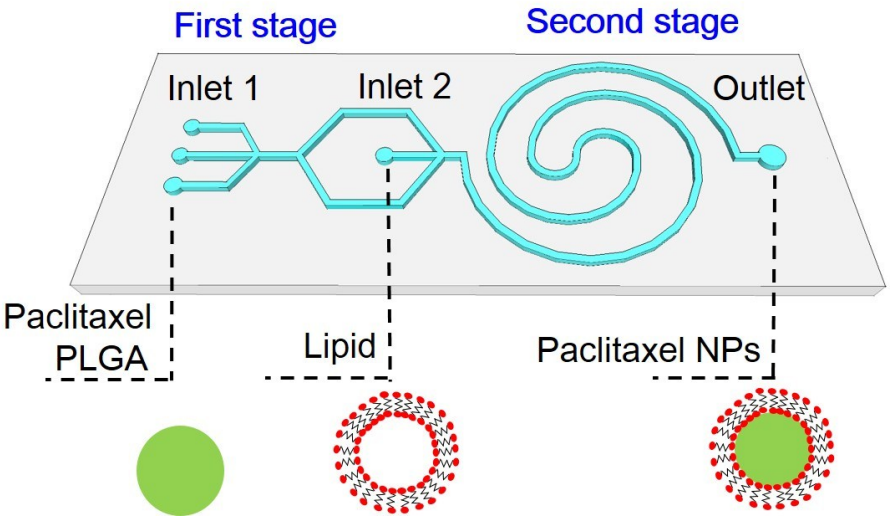

Figure S5 Schematic of the microfluidic chip for fabrication of paclitaxel-nanoparticles (NPs).

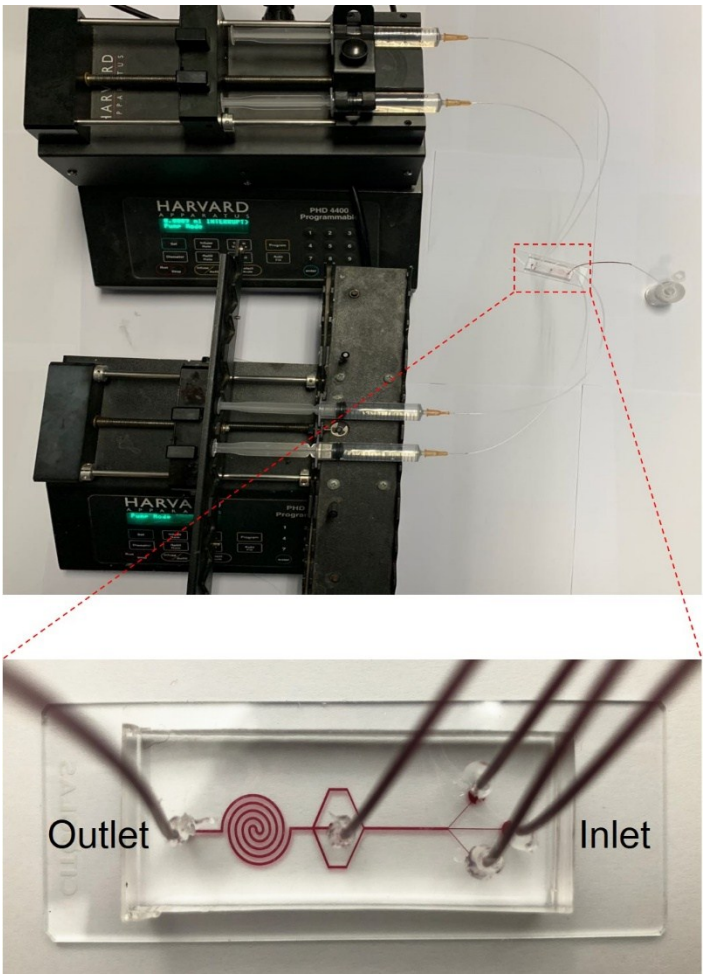

Figure S6 Photographs of the experimental setup and a two-stage microfluidic chip.

Characterization of rigid Paclitaxel-laden nanoparticles.

We investigated the cytotoxicity of Paclitaxel nanoparticle into the cell culture medium in co-cultured Hela cells and HUVECs by vessel network chip. In our previous work, rigid NPs were more easily to cross membranes and dramatically improved the cellular uptake efficiency.[3, 7] The microfluidic device for generation of rigid paclitaxel-laded nanoparticles consisted of two stages with four inlets and one outlet. The on-chip generation of rigid paclitaxel-laded nanoparticles fabrication was shown in Supplementary Information. Particle size plays an important role in determining the drug release behaviour of the paclitaxel-loaded nanoparticles as well as their fate after administration. Smaller particles tended to accumulate in the tumour sites due to the facilitated extravasation and a greater internalization was also observed. After fabrication of paclitaxel nanoparticles by microfluidic chip, we use transmission electron microscopy (TEM) and dynamic light scattering (DLS) to characterize their size, structure, and surface charge. TEM observation clearly shows that nanoparticles are spherical in shape and have a core-shell structure with PLGA and paclitaxel in the inner and lipid in the outer (Fig. S7a). From the results of DLS, the average diameter of the nanoparticles is 70 nm and the polydispersity index is 0.243 (Fig. S7b-d). There is no significant difference of the same paclitaxel-loaded nanoparticle in the particle size and polydispersity coefficients after three days. We also use DLS to measure the surface charge of hybrid nanoparticles. The zeta potential of nanoparticles is 35.6 mV. In order to make a better comparison with free drugs, we measure the entrapment efficiency (EE) of Paclitaxel. The encapsulation efficiency of paclitaxel into nanoparticles is 98.0 % (Fig. S8).

Dynamic light scattering (DLS, Zetasizer 3000HS, Malvern Instruments Ltd.) was used to measure the hydrodynamic diameter and zeta potential of paclitaxel-nanoparticles. 1 mL paclitaxel-nanoparticles suspension was placed in a DLS cuvette and measured with detection optics arranged at 173°. Three measurements were performed on each sample. For the stability study, 100 μL PBS was added to 900 μL paclitaxel-nanoparticles suspension. After mixing, the size and polydispersity index

(PDI) of the NPs in PBS was measured using DLS on the same day as well as subsequent days. For measurement of zeta potential, 700 μL of nanoparticle suspension in a disposable folded capillary cell was analyzed by DLS. At least triplicate measurements at 25 °C were performed on each sample. When the zeta potential is larger than ± 30 mV, the colloidal solution is considered as stable. The structure of rigid Paclitaxel-ladded nanoparticles was observed under transmission electron microscopy (TEM, FEI Tecnai T20). Carbon-coated copper grids were covered with 10 μL of nanoparticle suspension for 5 min followed by negative staining with 2% (w/v) phosphotungstic acid aqueous solution for 10 min. Excess stain was carefully removed using filter paper, and the copper grids were air-dried for 12 h. The dried grids with attached lipid−polymer nanoparticles were observed at an accelerating voltage of 200 kV by TEM.

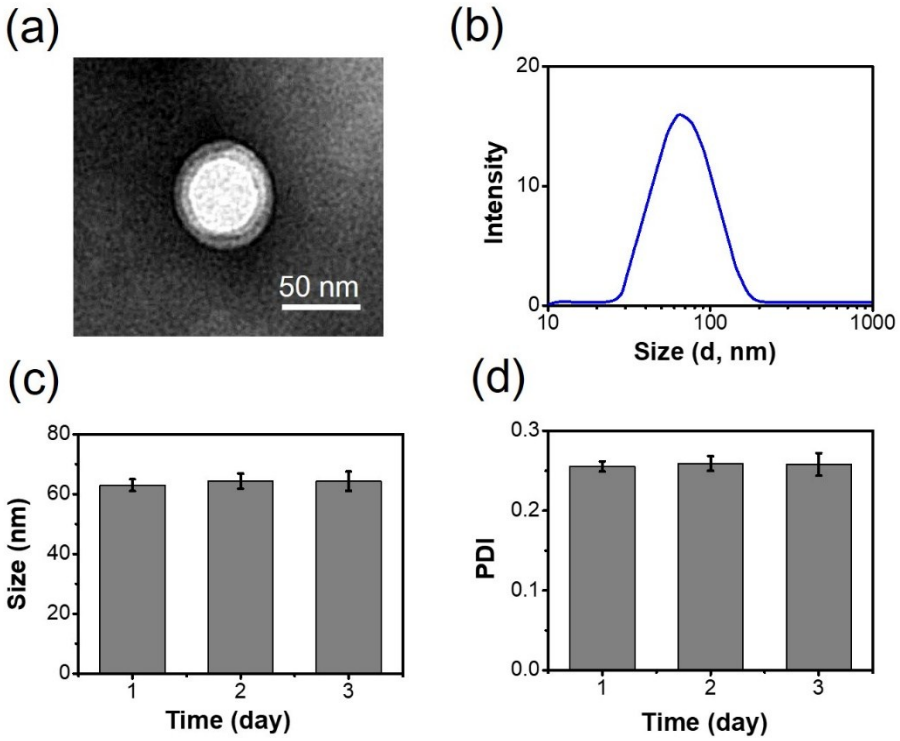

Figure S7 Characterization of paclitaxel nanoparticles. (a) TEM images of the paclitaxel nanoparticles. (b) Typical size distribution of paclitaxel nanoparticles. (c) Size and (d) polydipersity of same paclitaxel nanoparticles generated during 3 days.

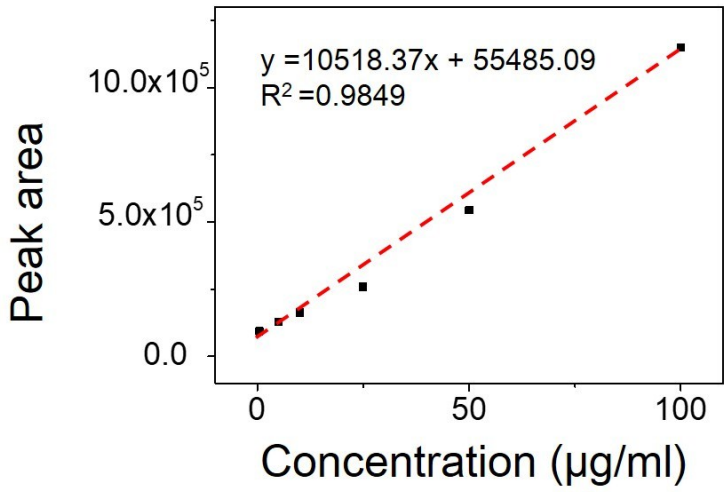

Figure S8 Drug entrapment efficiency in paclitaxel nanoparticles. The standard curve of paclitaxel.

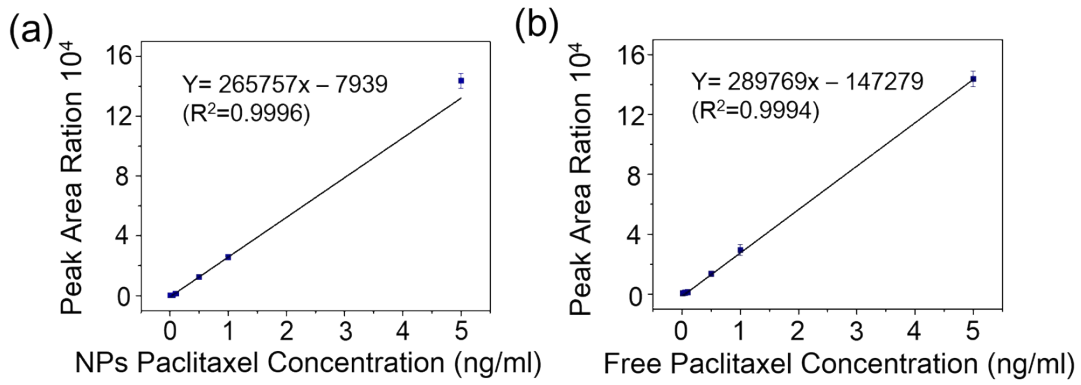

Figure S9 Calibration curve of NP-paclitaxel (a) and free-paclitaxel (b) in cell culture medium by MS (fragment ion 308.991).

Supplementary References

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