Supporting Information

Ultrasound-degraded serum albumin nanoplatform for *in situ* controlled drug release

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EXPERIMENTAL SECTION

Materials: glutaraldehyde (GA), 4,4'-(diazene-1,2-diyl)bis(4-cyanopentanoic acid) (ACVA), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), BSA, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 3-(4,5-dimethyl- 2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) trichloroacetic acid, sodium azide (NaN_3), chlorpromazine hydrochloride (CPZ) and colchicine (CC) were purchased from Energy Chemical Co., Ltd. And Nystatin was purchased from Macklin Co., Ltd. Trypsin (from bovine pancreas) was purchased from Aladdin Co., Ltd. Fetal bovine serum (FBS), phosphate buffered saline (pH 7.4), digestive enzyme (including EDTA), Dulbecco's modified Eagle's medium (DMEM), calcein acetoxymethyl ester (AM), propidium iodide (PI), trihydrochloride trihydrate (Hoechst 33342) and LysoTracker™ Red DND-99 were purchased from

Thermo Fisher Scientific Inc Co., Ltd. Deionized (DI) water used in all experiments was from Milli-Q system. All the reagents used in the experiments were of analytical grade and used directly without further purified.

Characterization: Transmission electron microscopy images of BSA NPs were obtained by HT7700 EXALENS. The absorbance spectra were measured using Agilent Technologies CARY 60 UV–vis spectrophotometer. The Fluorescence spectra were recorded using VAEIAN CARY Eclipse fluorescence spectrophotometer. The dynamic light scattering measurements were conducted by Malvern Zetasizer (Nanozs90). Instruments used in cell imaging tests were carried out on FV1000-IX81 confocal microscopy (Olympus, Japan). WED-100 ultrasound treatment apparatus (Welld, China) was used as the ultrasound trigger source. The circular dichroism spectra were carried on a circular dichroism spectrometer (J-1500, Jasco).

Preparation of BSA NPs: BSA NPs were prepared by a desolvation method. ACVA (50 mg, 178 μmol) was dissolved in 2 mL anhydrous dimethyl sulfoxide, and then EDC (50 mg, 0.26 mmol) and NHS (80 mg, 0.70 mmol) were added in sequence. The mixture was stirred for 2 h at room temperature. BSA (90 mg, 1.35 μmol) and DOX (6 mg, 10.35 μmol) were first dissolved in DI water (3 mL) under vigorous stirring (1 h). The pH of obtained solution was adjusted to about 9.0 with 0.1 M NaOH. Then 8 ml ethanol was added to above BSA solution dropwise at a rate of 4 mL min^{-1}. ACVA cross-linking solution (80 μL, 7.12 μmol) was slowly dropped into the mixture which was maintained stirring for overnight at room temperature. The obtained BSA–ACVA–DOX NPs were purified by centrifugation at 12000 rpm

for 15 min and washed with DI water for three times to eliminate the ethanol, free ACVA and DOX. Finally, the BSA NPs were dried by freeze dryer for further use. The BSA-ACVA NPs were synthesized with the same method of BSA–ACVA–DOX NPs with only BSA (90 mg) dissolved in DI water (3 mL). The BSA–GA–DOX NPs were synthesized using the same method with 100 μL of 8% GA instead of ACVA cross-linking solution.

Drug Loading and Encapsulation Efficiency: To determine the drug loading and encapsulation efficiency, the standard absorption curve of DOX with different concentrations in PBS were firstly tested. BSA–ACVA–DOX NPs (5 mg) were suspended in 10 mL of 1 mg mL^{-1} trypsin solution and the mixture was stirred overnight at 37 °C. The supernatant was filtered and transferred to a 100 mL volumetric flask, then the DOX content was measured using UV–vis spectroscopy at 479 nm. Drug loading and encapsulation efficiency were calculated as follows:

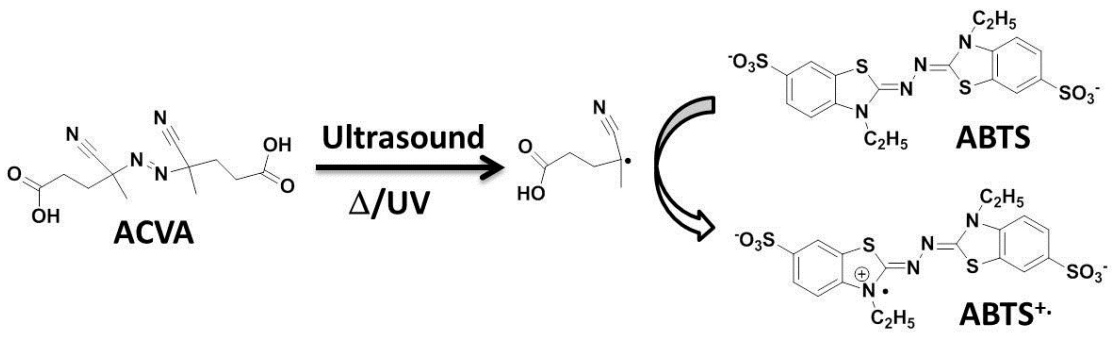
$$Drug\ loading(\%) = \frac{Mass\ of\ DOX\ Encapsulated\ (ME)}{Mass\ of\ DOX\ Encapsulated\ (ME) + Mass\ of\ BSA(MBSA)} \times 100$$

$$Encapsulation\ efficiency(\%) = \frac{Mass\ of\ DOX\ Encapsulated\ (ME)}{Mass\ of\ DOX\ Added} \times 100$$

In Vitro **Ultrasound Triggered Release of DOX:** Ultrasound-responsive DOX release profile of BSA–ACVA–DOX NPs and BSA–GA–DOX NPs were measured respectively. First, the BSA NPs solution (1 mL, 1 mg mL^{-1}) was transferred into a 1 mL centrifuge tube, which was covered under an ultrasound probe. The gap between the centrifugal tube and ultrasound probe was filled with specific ultrasound

couplants. The ultrasound (1 MHz) with different intensities and durations was used to trigger the release of DOX. After the ultrasound irradiation, the solution was centrifuged at 12000 rpm for 15 min. The supernatant was collected and the absorbance of DOX at 479 nm was measured by UV–vis spectrometer. The DOX concentration in the release medium was calculated according to the standard curve (Fig. S2). For the ultrasound-triggered DOX release, the BSA–ACVA–DOX NPs were irradiated by ultrasound at regular intervals (5 h). Moreover, pock tissues with different thickness (0, 0.5, 1.0, and 2.0 cm, from local supermarket) were placed between the centrifugal tube and ultrasound probe to simulate the ultrasound-triggered drug release in deep solid tumor.

Free Radicals Detection by $ABTS^{+}\bullet$: The ABTS probe was used to measure the free radicals generated by ACVA upon different intensities and durations of ultrasound irradiation. The ACVA solution (0.5 mg mL^{-1}, 0.5 mL) and ABTS solutions (2 mg mL^{-1}, 0.5 mL) were mixed together and transferred to a centrifuge tube. Then ultrasound irradiation was applied to the mixed solution. Afterward, the absorbance of green $ABTS^{+}\bullet$ solution ranging from 600 to 900 nm was recorded by the UV–vis spectrometer.



***In Vivo* Cellular Uptake and Cell Image:** Human breast cancer cells (MCF-7) were purchased from Institute of Basic Medical Sciences (IBMS) of Chinese Academy of Medical Sciences. MCF-7 cells were cultured in the DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. For confocal images, the cells were seeded in confocal culture dishes or cell culture flask and incubated under an atmosphere of 5% CO_2 at 37 °C for 24 h. The BSA–ACVA–DOX NPs were incubated with MCF-7 cells for 1 h with Hoechst33342 and LysoTracker™ Red DND-99 (Thermo Fisher Scientific) to label cell nuclei and lysosomes, respectively. The cells were treated with or without ultrasound irradiation (1 MHz, 2 W cm^{-2}) for different durations and then recorded under the confocal laser scanning microscope. Moreover, the MCF-7 cells were incubated for another 24 h, then stained with both AM and PI in confocal culture dishes for 30 min, and finally observed by the confocal laser scanning microscope. For cell uptake inhibition study, the cells were seeded in confocal culture dishes. The cells were washed with PBS and preincubated with the following endocytic inhibitors in complete medium for 1 h at 37°C: sodium azide (NaN3, 10 mM), chlorpromazine hydrochloride (CPZ, 10 mg/mL), colchicine (CC, 10 mg/mL) and nystatin (25 mM). For temperature pathway study, cells were pretreated at 4°C for 30 mins.The cells were further stained with BSA–ACVA–DOX NPs in DMEM medium with 1 h and recorded by the confocal laser scanning microscope. The software ImageJ was used to calculate the draw fluorescence intensity and count living or dead cells in images.

***In Vitro* Cytotoxicity by MTT Assay:** The MTT assay was carried out to test the relative cell viability. MCF-7 cells were seed in the 96-well microplates (Nunc, Denmark) with a density of 1×10^4 cells per well which were then incubated at 37 °C

for 24 h. Fresh medium containing different concentrations of the BSA NPs were added for 1 h more incubation with cells. After washing twice with PBS (pH 7.4, 100 mM), the cells were re-cultured in fresh medium, then dealt with ultrasound irradiation (1 MHz, 2 W cm^{-2}) for different durations (1 min and 3 min), and incubated at 37 °C for 24 h. As control, cells dealt with only BSA NPs, ultrasound irradiation and fresh medium respectively were tested. MTT solutions in DMEM (100 μL, 0.5 mg mL^{-1}) were added to each well, and after 4 h incubation at 37 °C the solutions in 96-wells plates were removed carefully and the formazan crystal were solved using 100 μL DMSO. The absorbance in each well was recorded by microplate reader (Thermo Fisher Scientific) at 570 nm (ODK) and 630 nm (OD). The cell viability was calculated by the following equation:

$$Cell\ viability(\%) = \frac{OD_{dye} - ODK_{dye}}{OD_{blank} - ODK_{blank}} \times 100$$

Effects of the ultrasound irradiation on biomolecules activity. The trypsin activity was detected by the method of National Standard of People's Republic of China (GB/T23527-2009). First, 2 mL trypsin solution (2 mg mL^{-1}) in PBS was pre-treated with ultrasound irradiation, mixed with 2 mL of substrate (10 g L^{-1} casein solution), and incubated at 37 °C for 10 min. The enzymatic reactions were ended by adding TCA solution (4 mL, 65.4 g L^{-1}). The suspension was centrifuged at 1000 rpm for 10 min. The supernatant was withdrawn and the absorbance at 275 nm was measured by UV–vis spectrometer. The trypsin activity was calculated by using the following equation:

$$X_1 = \frac{A_1 \times V_1 \times 4 \times N}{m} \times \frac{1}{10}$$

where X_1 was trypsin activity (U g^{-1}), A_1 was the enzyme activity of the sample on the standard curve (U mL^{-1}), V_1 (mL) was the volume of trypsin solution, N was the dilution ratio, and *m* (g) was the weight of trypsin.

The ultrasound irradiation (1 MHz, 2 W cm^{-2}) was applied to the CT-DNA solution (1 mL, 1 mg mL^{-1}) and BSA solution (1 mL, 1 mg mL^{-1}) solutions, respectively. The absorbance spectra, fluorescence spectra and circular dichroism spectra were recorded, respectively.

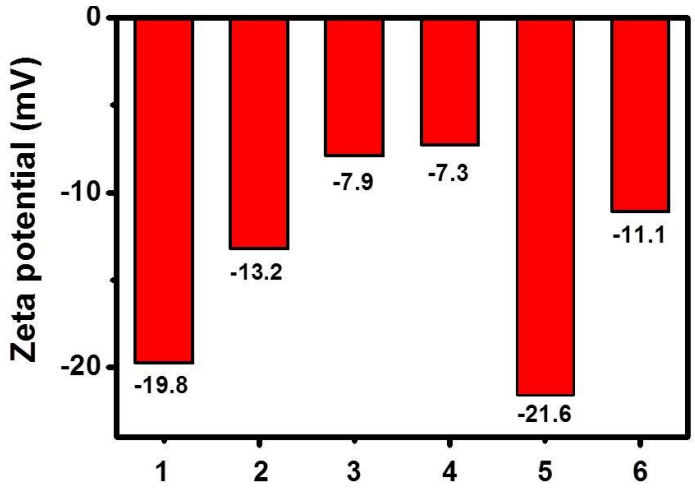


Fig. S1 Zeta potential of 1) BSA–ACVA-DOX NPs, 3) BSA–GA–DOX NPs, and 5) BSA–ACVA NPs; and zeta potential changes of 2) BSA–ACVA-DOX NPs, 4) BSA–GA–DOX NPs, and 6) BSA–ACVA NPs after ultrasound irradiation (1.0 MHz, 2 W cm^{-2} for 10 min).

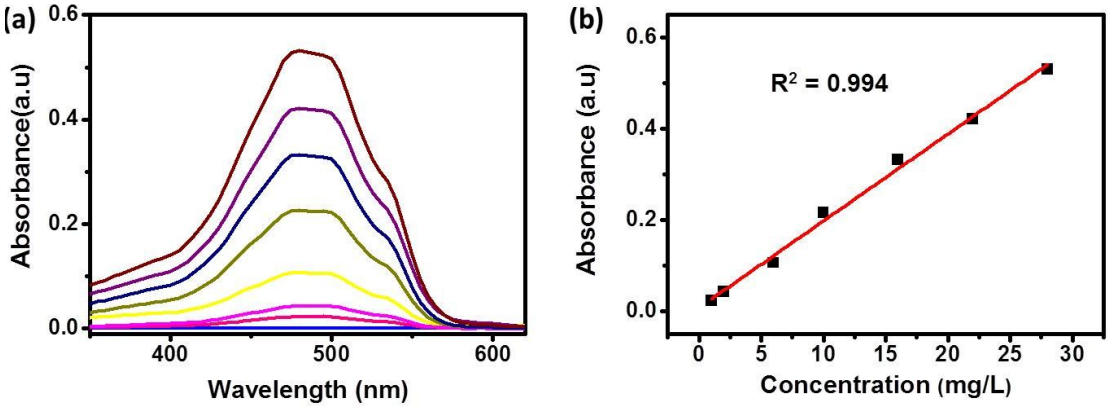


Fig. S2 (a) UV–vis absorption spectra of DOX at different concentrations; (b) The linear relationship between DOX concentration and absorption intensity at 479 nm.

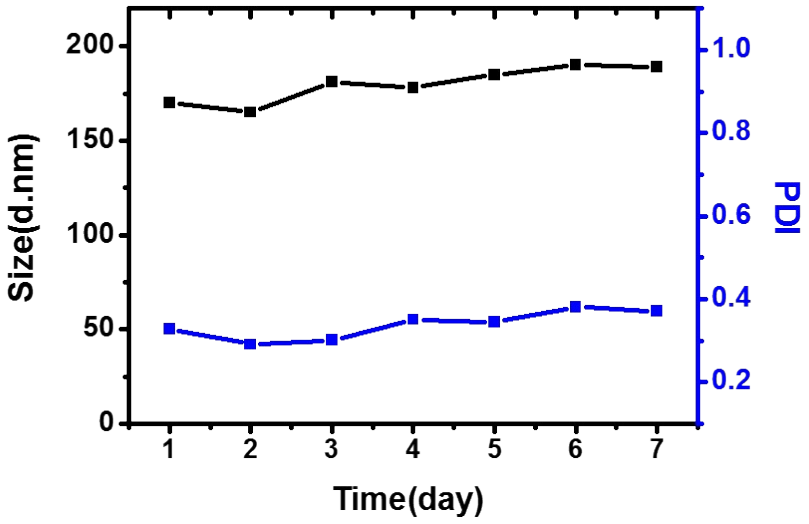


Fig. S3 Size and PDI for BSA–ACVA–DOX NPs in PBS (pH 7.4, 100 mM).

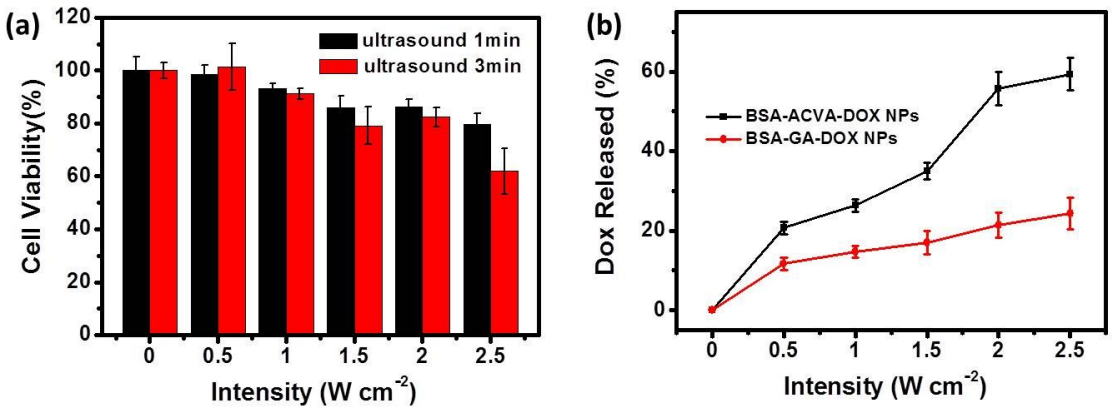


Fig. S4 (a) Cell viability of different ultrasound intensities (0, 0.5, 1.0, 1.5, 2.0, 2.5 W cm^{-2}) for 1 min (black bar) and 3 min (red bar). (b) Release percentages of BSA–ACVA–DOX NPs and BSA–GA–DOX NPs using ultrasound irradiation of different intensities (1.0 MHz for 3 min).

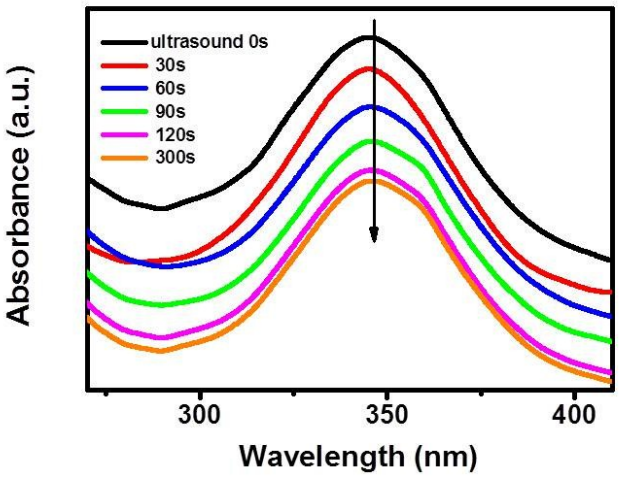


Fig. S5 The absorption of ACVA (500 μg mL^{-1}) under different ultrasound irradiation time (0, 30 ,60, 90, 120 and 300 s).

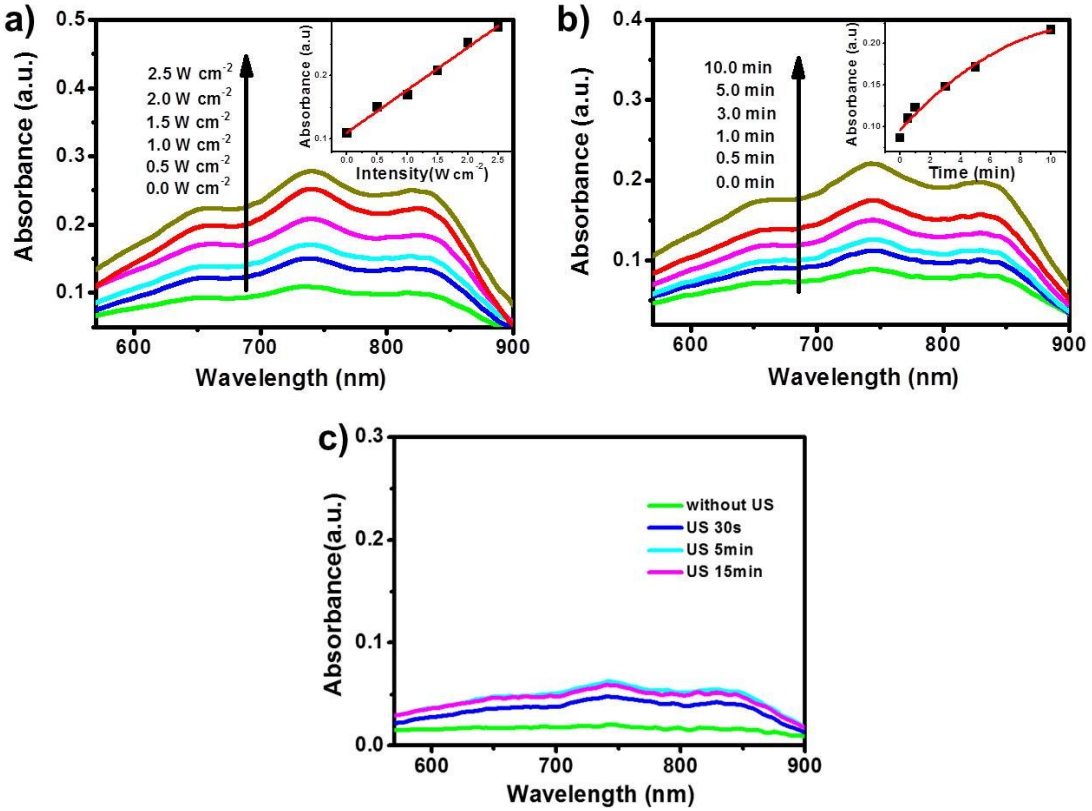


Fig. S6 UV-vis absorption spectra of $ABTS^{+\cdot}$ in the presence of different irradiation intensity (a) and duration (b), respectively; (c) UV-vis absorption spectra of ABTS in the presence ultrasound.

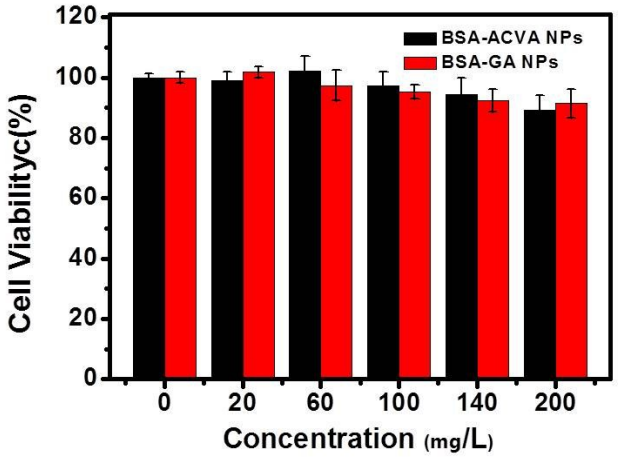


Fig. S7 Cell viability incubated with different concentrations of BSA–ACVA NPs (black bar) and BSA–GA NPs (red bar) in the absence and the presence of ultrasound irradiation (1.0 MHz, 2 W cm^{-2}).

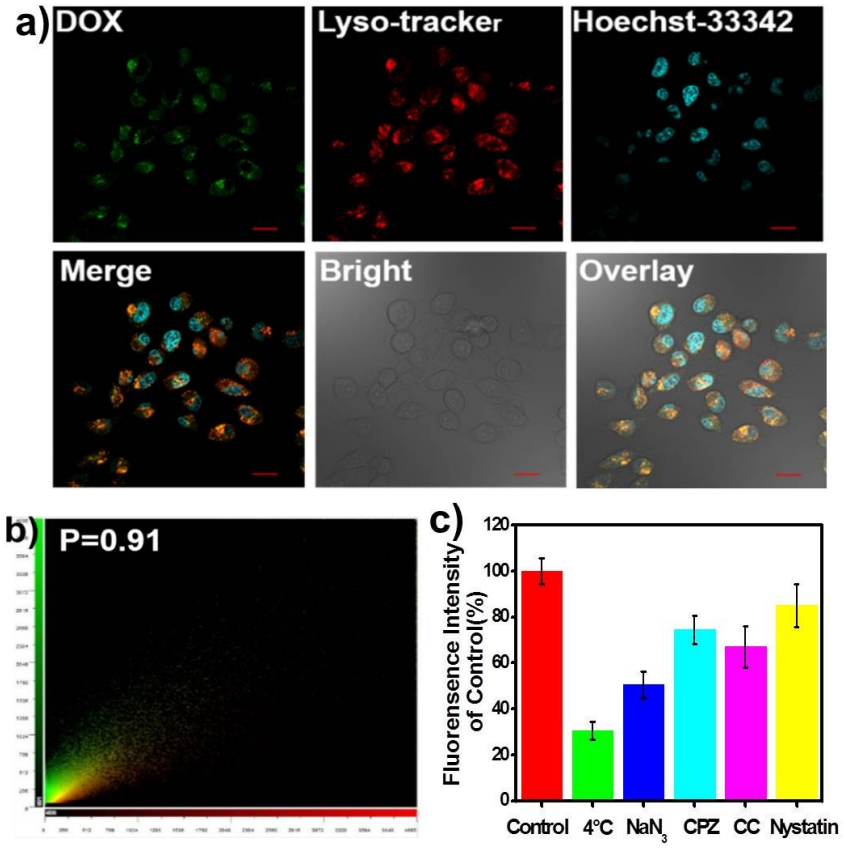


Fig. S8 (a) Confocal images of BSA–ACVA–DOX NPs incubated with MCF-7 cells. (b) Subcellular colocalization correlation coefficient with Lysotracker Red. (c) Cellular uptake pathway study of BSA-ACVA-DOX NPs by MCF-7 cells using specific endocytosis inhibitors and conditions.

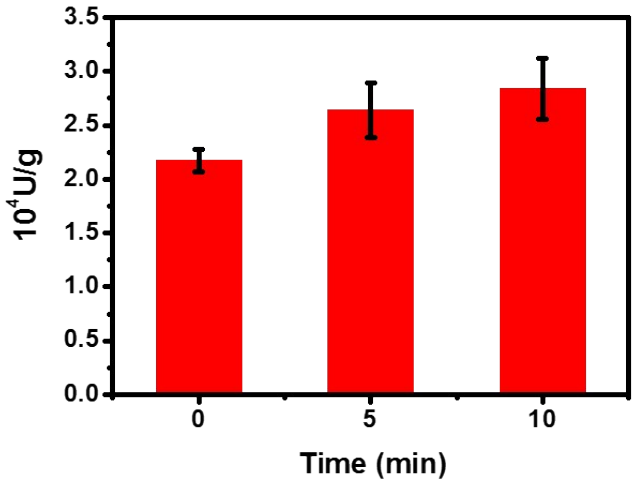


Fig. S9 The activity of trypsin after different ultrasound irradiation duration (0, 5, and 10 min, 1 MHz, 2 W cm^{-2}).

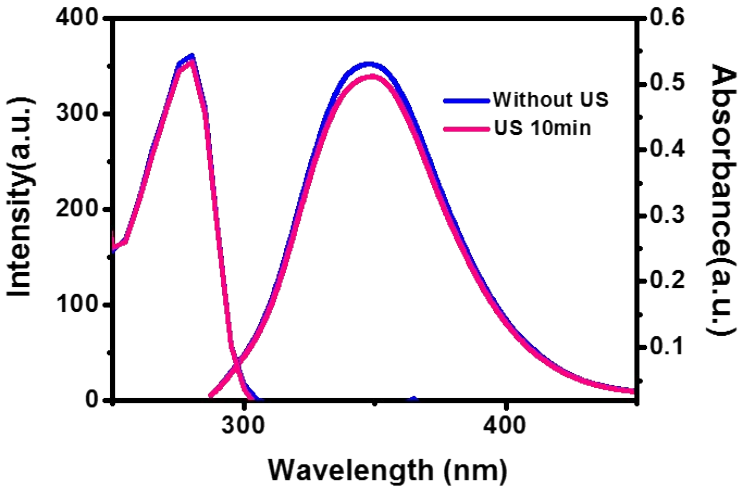


Fig. S10 Fluorescence spectra and UV-vis absorption spectra of BSA with and without 10 min ultrasound irradiation (1 MHz, 2 W cm^{-2}).

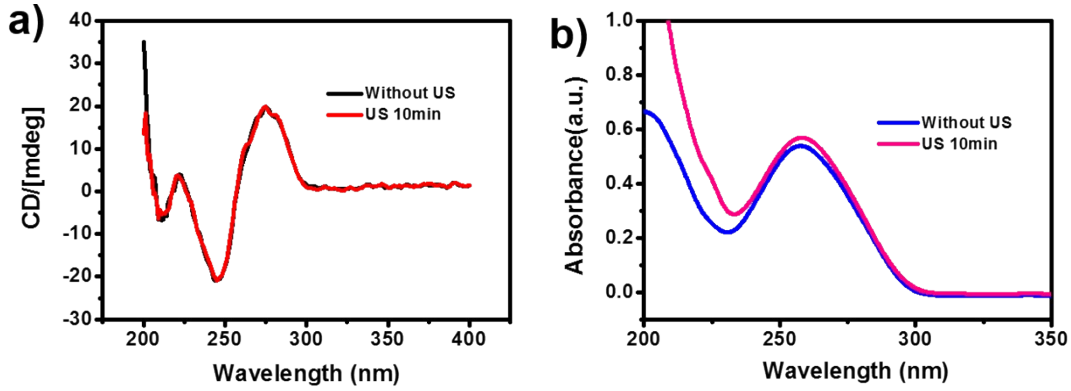


Fig. S11 (a) Circular Dichroism spectra and (b) UV-vis absorption spectra of CT-DNA with and without 10 min ultrasound irradiation (1 MHz, 2 W cm^{-2}).