Supplementary information

A cancer cell specific targeting nanocomplex for combination of mRNA-responsive photodynamic and chemo-therapy

Xiaolong Zhang[a,b], Xionghong Tan[c], Da Zhang[a,b], Naishun Liao[a,b], Youshi Zheng[a,b], Aixian Zheng[a,b], Yongyi Zeng[a,b,d], Xiaolong Liu[a,b],* and Jingfeng Liu[a,b,d]*

a. The United Innovation of Mengchao Hepatobiliary Technology Key Laboratory of Fujian Province, Mengchao Hepatobiliary Hospital of Fujian Medical University, Fuzhou 350025, P. R. China;

b. The Liver Center of Fujian Province, Fujian Medical University, Fuzhou 350025, P. R. China;

c. Fujian Institute of Research on the Structure of Matter Chinese Academy of Sciences, Fuzhou, Fujian 350002, P. R. China.

d. Liver Disease Center, The First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, P. R. China.

Corresponding author: E-mail Address: xiaoloong.liu@gmail.com Or E-mail Address: drjingfeng@126.com.

Experimental Section

Materials: Chlorin e6 (Ce6), doxorubicin (DOX), N, N'-Dicyclohexylcarbodiimide (DCC), N-hydroxy succinimide (NHS), disodium of 9,10-anthracenediyl-bis(methylene) dimalonic acid (ABDA), 2',7'-dichlorodihydr fluorescein diacetate (DCFH-DA), 4',6-diamidino-2-phenylindole (DAPI), protamine, HAase and BSA (Bovine Serum Albumin) were purchased from Sigma-Aldrich. Hyaluronic acid (HA, 90 kDa) was purchased from Freda Biochem Co., Ltd. (Shandong, China). Deoxyribonuclease I (DNase I) was purchased from Solarbio Science and Technology Co. Ltd. (Beijing, China). Cell Counting Kit (CCK8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Live-Dead Viability/Cytotoxicity Kit (Calcein AM/EthD-1) was purchased from Life Technologies, Inc. DNA oligonucleotides were synthesized and purified by Sangon Biotechnology Co., Ltd (Shanghai, China). The sequences of DNA molecular beacon (MB) used were listed as follows: BHQ2-MB: 5'-NH2-

ACGACGCCAGGGAGAACAGAAACCGTCGT-BHQ2-3'; DNA target: 5'-GTTTCTGTTCTCCCTGG-3'; TK1 mismatched target: 5'-GTTTATGTGCTACCTGG-3' and c-myc target: 5'-CCTCAACGTTAGCTTCACCAA-3'; control MB (mismatched TK1 probe, BHQ2-ctrl-MB): 5'-NH2-ACGACGCCACGGAGTACAGTAACCGTCGT-BHQ2-3'. Cell culture products, unless mentioned otherwise, were purchased from Thermo Fisher Scientific (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). All other reagents of analytical reagent grade were purchased from Sinopharm Chemical Reagent Co. Ltd. (China) and used without further purification. Ultrapure water prepared from a Millipore water purification system (18.2 M·Ω resistivity) was used in all runs.

Cell Culture: HeLa cells and HUVEC cells were cultured in RPMI medium 1640 and NIH 3T3 cells were cultured in DMEM medium. All cell lines were supplemented with 10% fetal bovine serum and 100 U/ml 1% antibiotics penicillin/streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 incubator.

Preparation of Ce6 modified MB (Ce6-BHQ2-MB): The modification of DNA with Ce6 was according to previous work.[1,2] In brief, an equal molecular amount of Ce6 (10 μmole), DCC and NHS were mixed in 250 μL N, N-Dimethylformamide (DMF) for activation reaction with 1 hour stirring. The activated Ce6 was then added to 5' end amine modified ssDNA (BHQ2-MB) in 250 μL 0.1 M pH 7.5 NaHCO3 solution for coupling by vigorously stirring overnight in the dark. The amount of Ce6 was 10 times more than DNA product in the coupling reaction to improve the coupling efficiency. To remove the uncoupled reagents, the product was precipitated by the mixture of 50 μL 2.0 M NaCl and 1.0 mL ethanol for four times, and the Ce6-BHQ2-MB was obtained. The conjugated DNA and Ce6 was quantified by measuring the absorbance at 260 and 404 nm. The characteristic absorption peak of DNA at 260 nm with a molar extinction coefficient of 6600 $M^{-1} \cdot cm^{-1}$ and Ce6 at 404 nm with a molar extinction coefficient of 1.1×10^5 $M^{-1}cm^{-1}$ were used to determine the concentration.[3] The Ce6-BHQ2-cMB was prepared at the same way instead with the BHQ2-ctrl-MB.

Responsive of DOX-MB with DNA target. To load DOX into the Ce6-BHQ2-MB, the DNA solution in PBS buffer (10 mM NaH_2PO_4/Na_2HPO_4 of pH 7.4, 10 mM NaCl, 10 mM $MgCl_2$) was heated at 80 °C for 5 min, cooled down to room temperature slowly, and

stored in the dark for at least 12 h to form MB structure. Certain amounts of DOX was incubated with the MB solution (10 μM) for at least 12 h to obtain the DOX-MB (the molar ratio of duplex to DOX was 0.5). Subsequently, DOX-MB (500 nM) was incubated with increasing concentrations of the DNA target (0, 25, 50, 100, 150, 200, 300, 400, 500, 750 nM) for 30 min at 37 °C. Fluorescence measurements were performed on an Agilent Cary Eclipse fluorescence spectrophotometer. After incubation, the fluorescence spectra were collected for Dox with scanning range from 510 to 700 nm under an excitation of 485 nm and for Ce6 with scanning range from 600 nm to 750 nm under an excitation of 404 nm.

Preparation of DOX-loaded nanocomplex. Nanocomplex was prepared by simply mixing of protamine, DNA and HA aqueous solutions. Briefly, 100 μL DOX-MB solution (10 μM) was first mixed with 200 μL HA solution (2 $mg \cdot mL^{-1}$) for 10 min. Then, 200 μL protamine solution (1 $mg \cdot mL^{-1}$) was dropwise added into the mixture and further incubated for 20 min to allow complete formation and stabilization of the nanocomplex. The mixed solution was purified by adding to Eppendorf tube with a 20 μL glycerol bed carefully deposited at the bottom and the nanocomplex was precipitated by repeated low-speed centrifugation (2000 rpm, 10 min)[4]. After discarding the supernatants, the HA-Pro-Ce6-DOX nanocomplex in the bottom of tube was resuspended in PBS buffer. The HA-protamine (HA-Pro), which prepared without Ce6-BHQ2-MB, HA-protamine-Ce6 (HA-Pro-Ce6), which prepared with Ce6-BHQ2-MB, and HA-Pro-ctrl-Ce6, which prepared with Ce6-BHQ2-cMB, were prepared at the same way as control.

Characterization of the nanocomplex. The average size, polydispersity (PDI), and zeta potential of the different components of nanocomplex were measured by DLS using a ZetaSizer Nano series Nano-ZS (Malvern Instruments Ltd, Malvern, UK). Transmission electron microscopy (TEM) was carried out on a JEOL model JEM-2010 transmission electron microscope. For TEM characterization, the sample was immobilized on copper grids and stained with 1% (w/v) phosphotungstic acid for 30 s. After air dry, the sample was observed by TEM. The Gel electrophoresis was used to confirm the loading of DNA into nanocomplex and evaluate the nuclease stability of DNA-loaded

nanocomplex. The sample (10 μL) was mixed with 6 × loading dye (2 μL) and analyzed using 3% agarose gel in 1 × TBE buffer. Gel electrophoresis was carried out at 110 V for 20 min and the gel was subsequently photographed using "ChemiDoc MP Imaging System" from Bio-rad.

ROS generation of Ce6-BHQ2-MB toward target DNA under 670 nm laser irradiation. ABDA was used as an indicator to assess $^1O_2$ generation.[2] The Ce6-BHQ2-MB was incubated with the completely matched target in PBS for 30 min at 37 °C. Then, 25 μM of ABDA solution was added and the photooxidation of ABDA was monitoring under the 670 nm laser irradiation with the power intensity of 0.2 W·cm^{-2} for 30 min at every 5 min. The absorbance change of ABDA was measured by an UV-vis spectrometer on a microplate reader (Spectra Max M5, Molecular Devices).

Release of Dox and activation of Ce6 of the nanocomplex with HAase treatment and DNA target incubation. The drug loaded nanocomplexes (HA-Pro-Ce6-DOX) were incubated with HAase (0.5 mg·mL^{-1}) in PBS at pH 5.5 for 2 h, and then incubated with target DNA (500 nM) with different incubation time. At scheduled time points, samples were centrifuged and the fluorescence of obtained supernatant was measured; afterwards, the release of DOX and activation of Ce6 were calculated according to their corresponding standard curves.

Cellular uptake and intracellular response to target mRNA. Cell uptake of nanocomplex was evaluated by using a LSM 780 confocal laser scanning microscopy (CLSM, Carl Zeiss, Boston, MA, USA). Hela cells and NIH3T3 cells were seeded onto a 35 mm glass-bottom Petri dish at a density of 1 × 10^5 cells per well at 37 °C and cultured for 12 h. The cells were then incubated with Opti-MEM containing the nanocomplex (500 nM MB) for 2 h. For inhibition tests, HA (10 mg·mL^{-1}) was pretreated and incubated for 2 h at 37 °C prior to treatment. Subsequently, the cells were washed twice with PBS and incubated with fresh medium for additional 4 h. After wash twice with PBS, the cells were fixed with 4% formaldehyde and stained with DAPI at room temperature for 10 min. Finally, the cells were washed and imaged by confocal microscope with 488 nm laser excitation for DOX (Em 550–590 nm), 405 nm laser excitation for DAPI (Em 430-480nm) and 405 nm laser excitation for Ce6 (Em

650–680 nm). To regulate the expression levels of TK1 mRNA, the HeLa cells were divided into three groups. One group of HeLa cells was treated with β-estradiol (10^{-8} mol/L) for 48 h to up-regulate the TK1 mRNA expression and the other group of HeLa cells was treated with tamoxifen (10^{-6} mol/L) for 48 h to down-regulate the TK1 mRNA expression. Afterwards, the cells were incubated with nanocomplex and imaged by confocal microscope as mentioned above.

Intracellular ROS detection. Intracellular ROS generation was measured by DCF-DA as the ROS fluorescence indicator. Hela cells were seeded in 96 wells plate at a density 1×10^4 for 12 h. Following incubation with different nanocomplex for 2h, the cells were washing and incubated of additional 4 h; then the DCF-DA was loaded into the cells for 30 min, afterwards, the cells were washed twice with PBS and then exposed to 670 nm laser for 15 min with the power intensity of 0.2 $W \cdot cm^{-2}$. The fluorescence images were acquired using a fluorescence microscope.

Cytotoxicity assay. The cytotoxicity of nanocomplexs were evaluated using CCK-8 assay according to the manufacturer's instructions. Hela cells and NIH3T3 cells were seeded into 96-well microtiter plates at a density of 1×10^4 cells per well in 100 μL culture medium and incubated at 37 °C with 5% CO_2 for 24 h. The Hela cells and NIH3T3 cells were divided into five groups in parallel, respectively. The cells were incubated with culture medium, HA-Pro, HA-Pro-Ce6, HA-Pro-Ce6-DOX at the concentration of 400 $\mu g \cdot mL^{-1}$ of HA-Pro and DOX at the concentration of 1 μM, respectively. After 2 h, the medium was replaced with fresh medium and the cells were further cultured for 4h; afterwards, the cells were irradiated with 670 nm laser (0.2 $W \cdot cm^{-2}$) for 15 min, and then further cultured for 48 h before the CCK8 assay. The cells without laser irradiation were used as control.

To further evaluate the therapeutic efficiency of our nanocomplex, the Live-Dead Viability/Cytotoxicity staining assay was carried out to the cells that were treated similarly as mentioned above. After the mentioned treatments, the cells were washed with PBS, and stained with 2.0 μM calcein AM and 4.0 μM EthD-1 for imaging the live and dead cells by using of a fluorescence microscope.

References

1 Z. Zhu, Z. Tang, J. a Phillips, R. Yang, H. Wang and W. Tan, *J Am Chem Soc*, 2008, **130**, 10856–10857.

2 D. Zhang, A. Zheng, J. Li, M. Wu, Z. Cai, L. Wu, Z. Wei, H. Yang, X. Liu and J. Liu, *Adv. Sci.*, 2017, 1600460.

3 B. Tian, C. Wang, S. Zhang, L. Feng and Z. Liu, *ACS Nano*, 2011, **5**, 7000–7009.

4 S. Wang, M. Cao, X. Deng, X. Xiao, Z. Yin, Q. Hu, Z. Zhou, F. Zhang, R. Zhang, Y. Wu, W. Sheng and Y. Zeng, *Adv. Healthc. Mater.*, 2015, **4**, 281–290.

Supplementary figures:

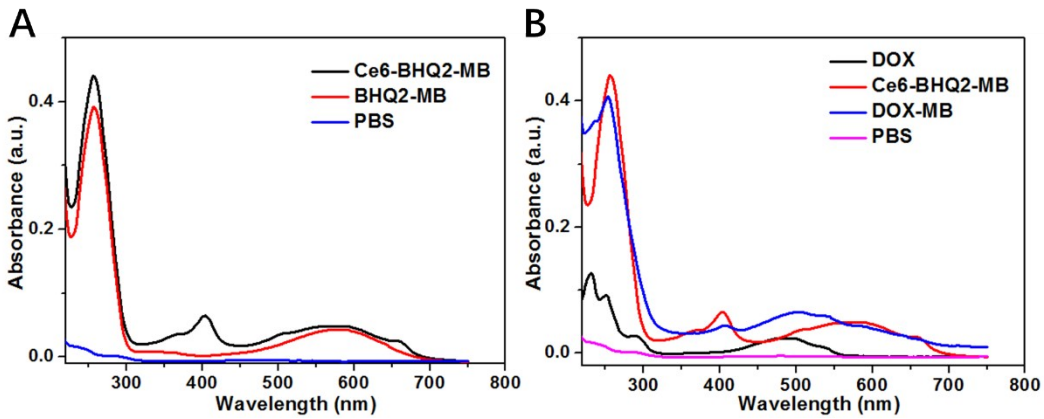

Fig. S1 (A) UV/Vis spectra of BHQ2-MB andCe6-BHQ2-MB. (B) UV/Vis spectra of free DOX, Ce6-BHQ2-MB and DOX-MB.

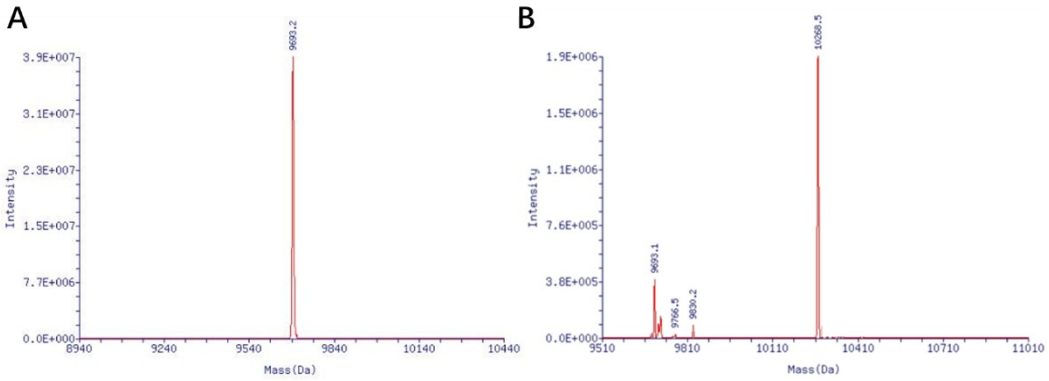

Fig. S2 The mass spectra of molecular beacon before (A) and after (B) coupling with Ce6.

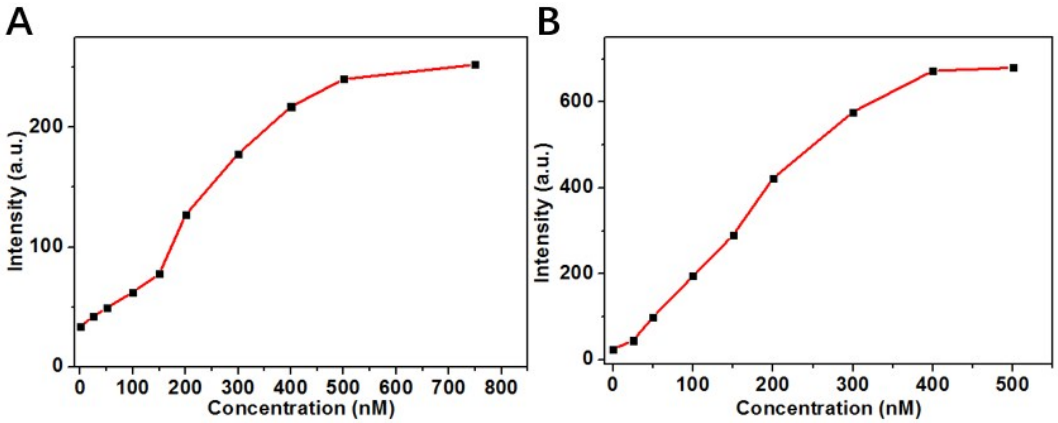

Fig. S3 (A) Fluorescence intensity curves of the DOX-MB in the presence of different concentration of targets (0, 25, 50, 100, 150, 200, 300, 400, 500, 750 nM) that were measured with 485 nm excitation (the emission fluorescence intensity was measured at 590 nm). (B) Fluorescence intensity curves of the Ce6-BHQ2-MB in the presence of different concentration of targets (0, 25, 50, 100, 150, 200, 300, 400, 500 nM) that were measured with 404 nm excitation (the emission fluorescence intensity was measured at665 nm).

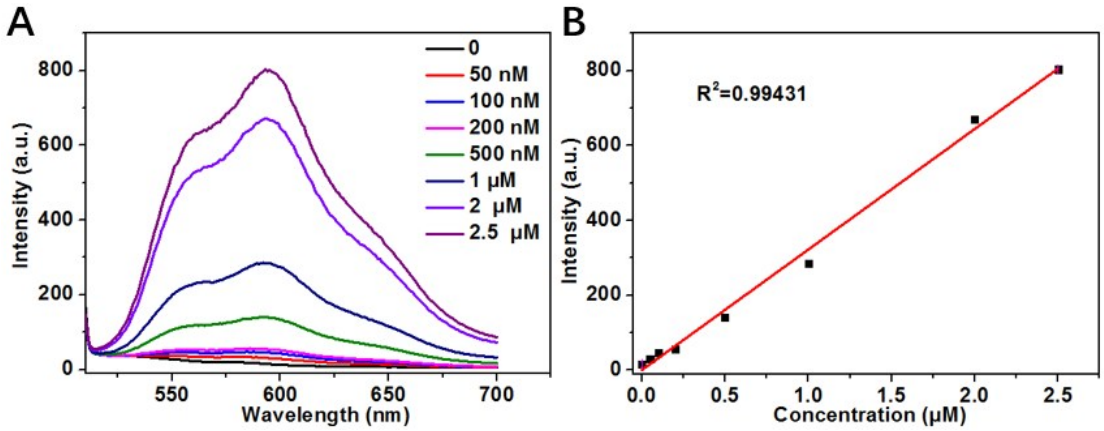

Fig. S4 The fluorescence emission spectra (A) and standard curve with linear calibration of DOX (B) at different concentrations.

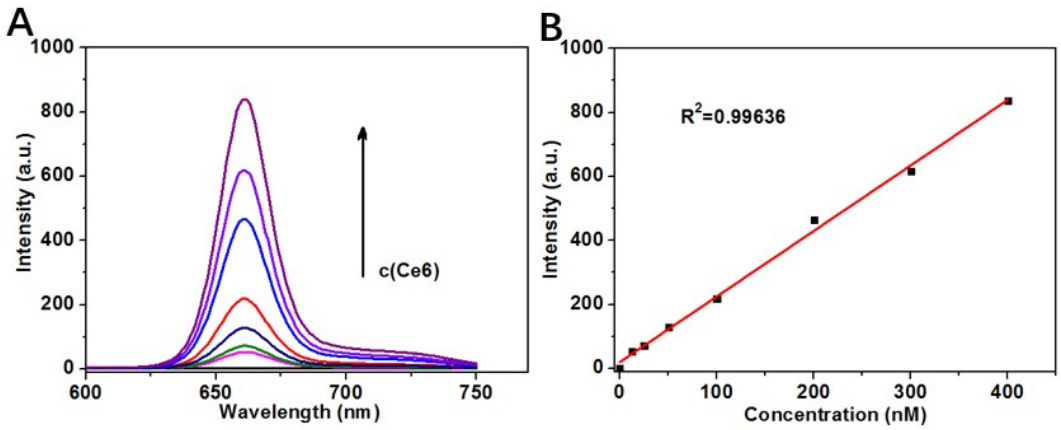

Fig. S5 The fluorescence emission spectra (A) and standard curve with linear calibration of Ce6 (B) at different concentrations (0, 25, 50,100, 200, 300, 400 nM).

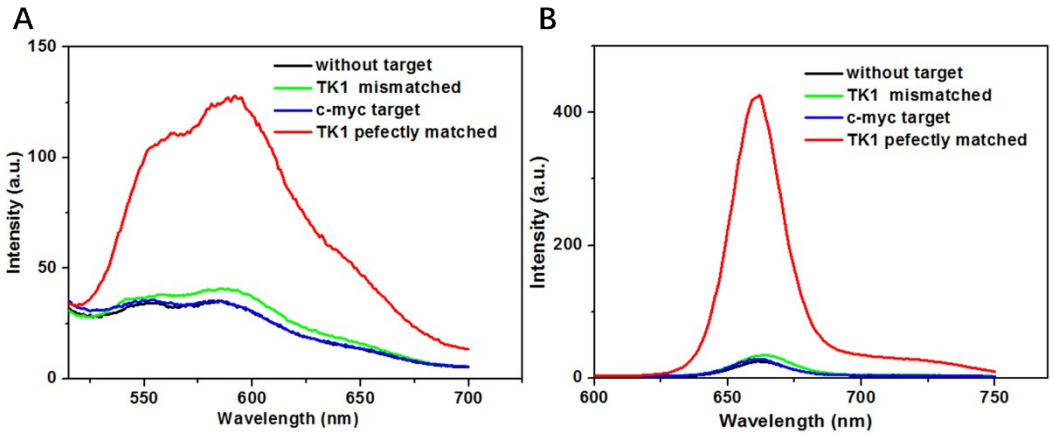

Fig. S6. (A) The fluorescence spectra of DOX-MB in the presence of different targets. (B) The fluorescence spectra of Ce6-BHQ2-MB with different targets. The concentration of the target is 200 nM.

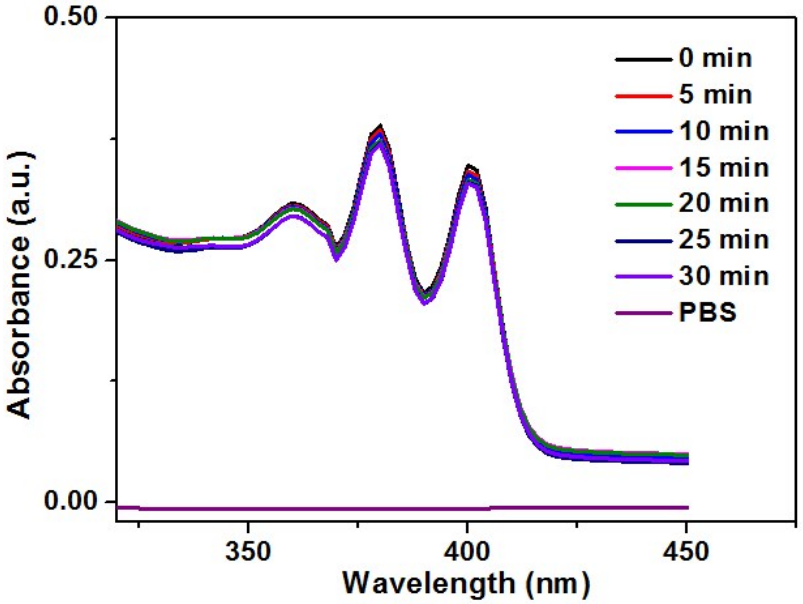

Fig. S7 The absorbance of 9,10-dimethylanthracene (ABDA, 25 μM) after photodecomposition by ROS generation upon 670 nm laser irradiation at 0.2 W·cm^{-2} in the presence of Ce6-BHQ2-MB without target.

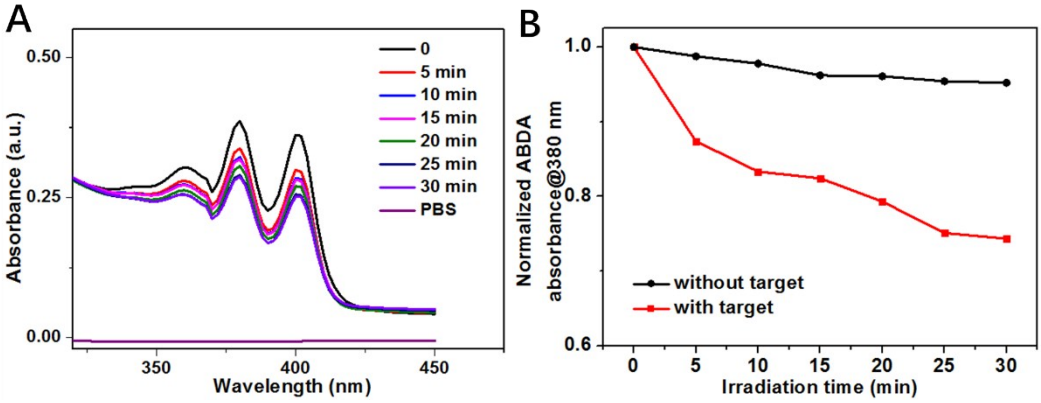

Fig. S8 (A) The absorbance of 9,10-dimethylanthracene (ABDA, 25 μM) after photodecomposition by ROS generation upon 670 nm laser irradiation at 0.2 W·cm^{-2} in the presence of Ce6-BHQ2-MB with target. (B) Normalized absorbance of ABDA at 380 nm during photodecomposition by ROS generation of Ce6-BHQ2-MB without target and with target.

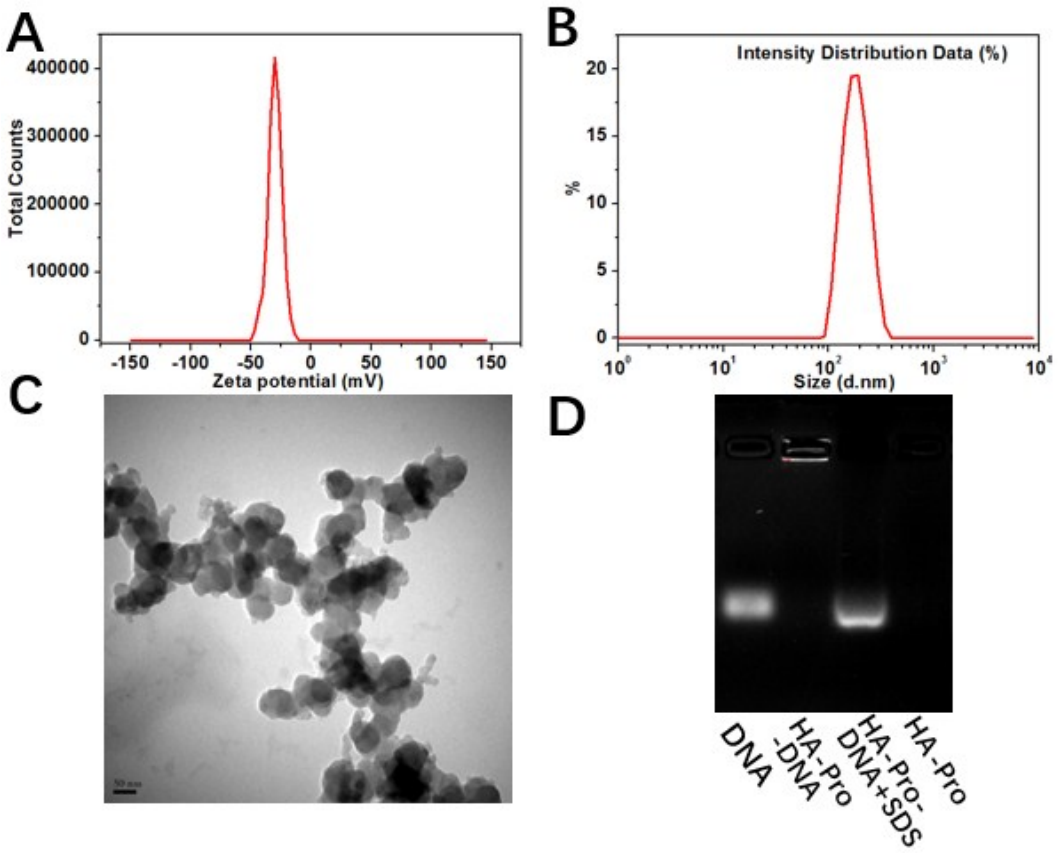

Fig. S9 Zeta potential (A), Size distributions (B) and TEM image of HA-Pro-DNA nanocomplex (C). The scale bar is 50 nm. (D) Gel electrophoresis was performed to prove the encapsulation of DNA in HA-Pro-DNA nanocomplex.

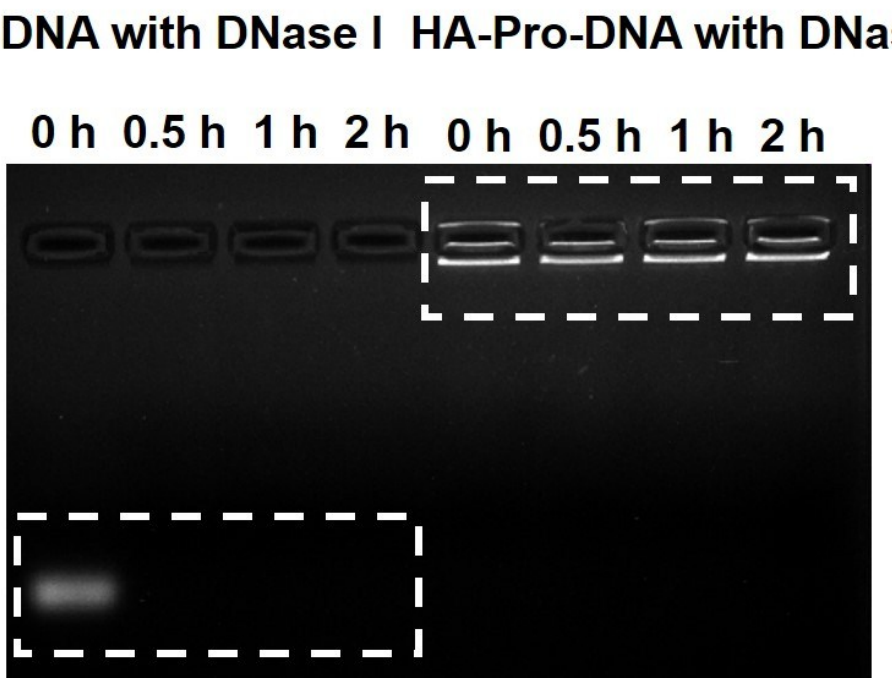

Fig. S10 The stability of free DNA and HA-Pro-DNA nanocomplex in the presence of DNase I (0.5 U·mL^{-1}) at different time.

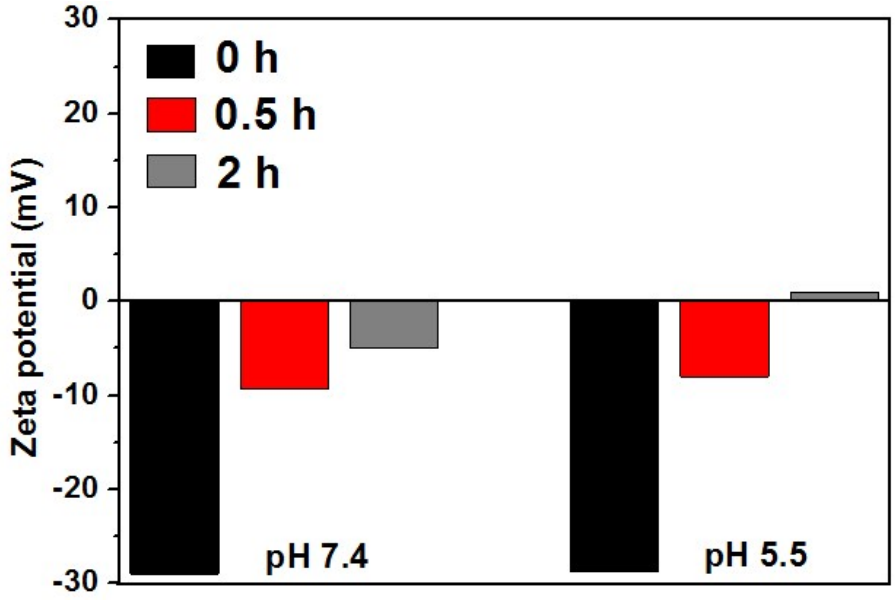

Fig. S11 Changes of the zeta potential of HA-Pro-DNA nanocomplex that were incubated with 0.5 mg·mL^{-1} HAase at different pH values for different time.

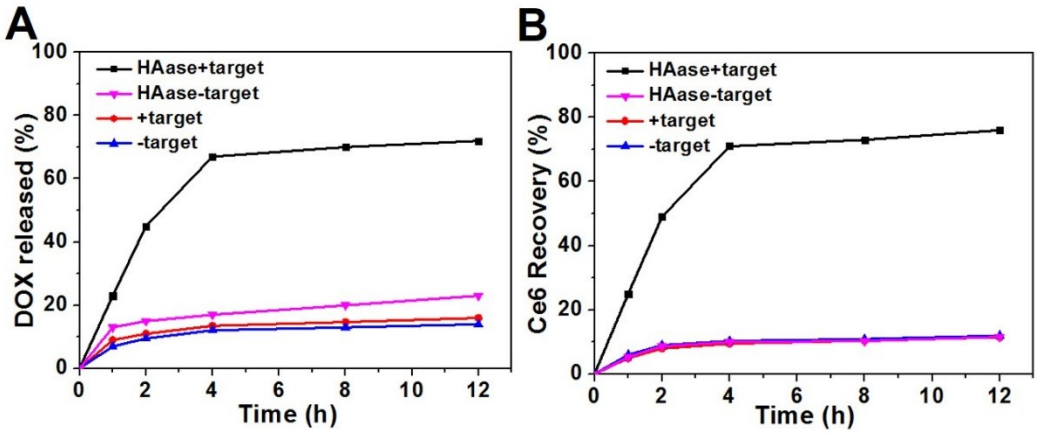

Fig. S12 Target responsive DOX release and Ce6 fluorescence recovery of HA-Pro-Ce6-DOX nanocomplex with and without HAase pretreatment.

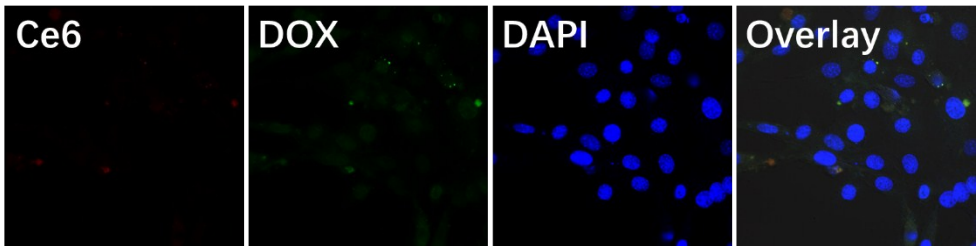

Fig. S13 Intracellular delivery of HA-Pro-Ce6-DOX to HUVEC cells observed by CLSM. The Ce6 was excited by 405 nm laser and Dox was excited by 488 nm laser, respectively.

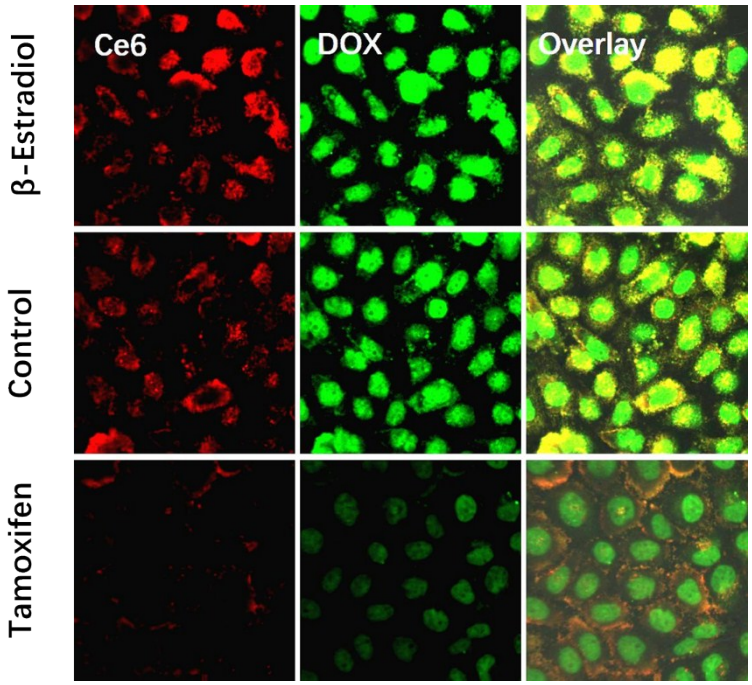

Fig. S14. Intracellular imaging of Ce6 and DOX that released from HA-Pro-Ce6-DOX by CLSM under different expression levels of TK1 mRNA. The upper panel were the β-estradiol-treated group that could upregulate the TK1 mRNA expression, the middle panel were the control group without treatment that has the moderate level of TK1 mRNA expression, and the bottom panel were the tamoxifen-treated group that could downregulate the TK1 mRNA expression. The Ce6 was excited by 405 nm laser and Dox was excited by 488 nm laser, respectively.

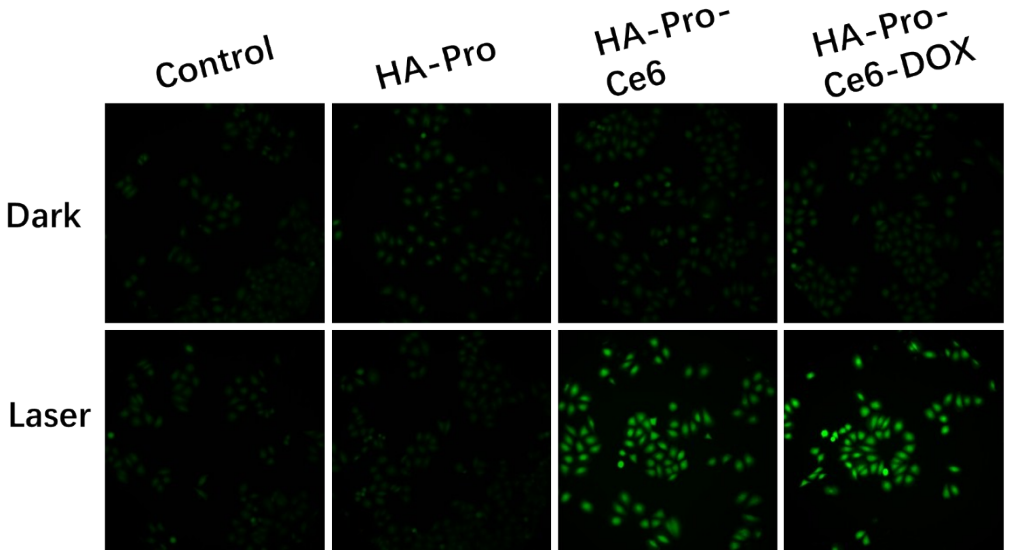

Fig. S15 The intracellular ROS detection (indicating by fluorescence intensity of DCF-DA) of Hela cells, that were incubated with HA-Pro, HA-Pro-Ce6 and HA-Pro-Ce6-DOX and then with or without laser irradiation.

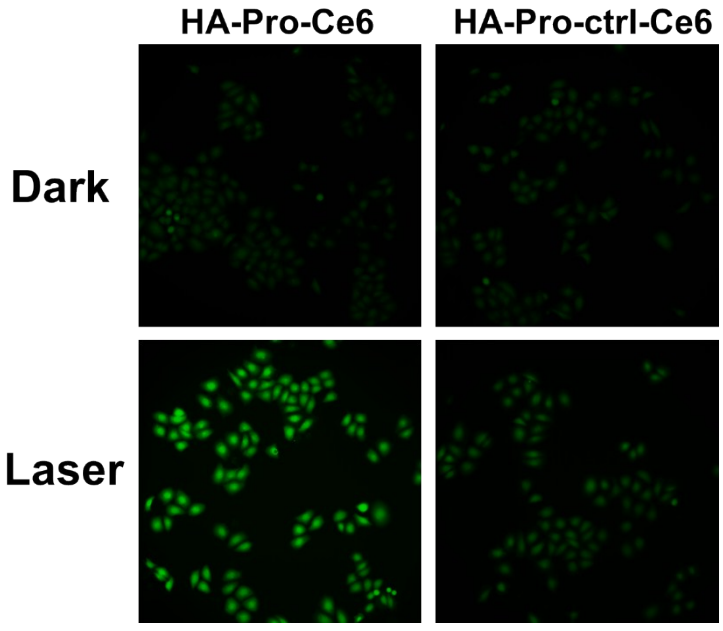

Fig. S16 The intracellular ROS detection of Hela cells, indicating by the fluorescence intensity of DCF-DA. The cells were incubated with HA-Pro-Ce6 (left column) and HA-Pro-ctrl-Ce6 (right column, the control nanocomplex), then with or without laser irradiation.

Supplementary tables:

Table S1 Physicochemical properties in water of the HA-Pro nanocomplex with different mass ratio of HA-Pro.

Formation	Pro (mg)	HA (mg)	Diameter (nm)	Zeta potential (mV)	PDI
HP0.5/1.0	0.5	0.25	101	+28.3	0.023
HP1.0/1.0	0.5	0.5	213	+10.4	0.048
HP1.5/1.0	0.5	0.75	538	+5.83	0.834
HP2/1.0	0.5	1	151	-29.9	0.09

Table S2 The encapsulation efficiency of DOX-MB in HA-Pro (2:1).

Formation	DOX-MB/100 μL	HA-Pro	Diameter (nm)	Encapsulation efficiency/%
HP-0.1	0.1 μM	2:1	153	99.1
HP-1	1 μM	2:1	151	98.3
HP-5	5 μM	2:1	150	97.1
HP-10	10 μM	2:1	155	92.1
HP-20	20 μM	2:1	152	83.7
HP-40	40 μM	2:1	148	71.3

Note: The encapsulation efficiency of DOX-MB was determined through absorbance values. The nanocomplex with the weight ratio of 2:1 of HA and protamine was used for encapsulation. The amount of DOX-MB loaded into the nanocomplex was determined according to the standard curve of Ce6.