Spatiotemporally Regulated Mitochondrial Genome Editing via Enzyme and NIR-Activated CRISPR/Cas9 nanoplatform

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Chemicals and Materials.

Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), were obtained from Sigma-Aldrich (MO, USA). $LnCl_3 \cdot 6H_2O$ (Ln = Tm, Yb, and Y) , NH_4F ，Oletic acid，1-octadecene (ODE, 95%) was purchased from Aladdin Industrial Co. Ltd. (Shanghai, China). Tetraethyl orthosilicate (TEOS), (3-aminopropyl)trimethoxysilane (APTES), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), (4-carboxybutyl) triphenylphosphonium bromide (TPP) and 4-maleimidobutyric acid N-hydroxysuccinimide ester（suflo-SMCC）were purchased Sigma-Aldrich. DMEM medium (high glucose), trypsin–EDTA solution, GelRed, poly(L-lysine, PLL) were purchased from Sangon Biotech (Shanghai, China). RNase free water，NEB 3.1 buffer, Cas9 protein, APE1 was bought from New England Biolabs (Ipswich, USA). Hoechst 33342 (C1027), Mito-Tracker Red CMXRos (C1035), Cell Counting Kit-8 (CCK-8), MMP assay kit with JC-1 (C2003), and ROS assay kit (S0033) , Calcein-AM/PI staining kit were all purchased from Beyotime Biotechnology Co. Ltd. (Shanghai, China). Fetal Bovine Serum (FBS) was purchased from Gibco (Waltham, USA). Annexin V-FITC/PI Apoptosis Detection Kit ere purchased from YEASEN Biotechnology Co. Ltd. (Shanghai, China). All DNA oligonucleotides were synthesized by Sangon Biotech (Shanghai, China). sgRNA was

purchased from GenScript Biotech Co. Ltd.(nanjing,China). The DNA and RNA sequences were provided in Table S1.

Instruments

Fluorescence spectra were measured using the F-7000 spectrophotometer (Hitachi, Japan). Kinetics fluorescence and in vitro cytotoxicity assays were performed with the TECAN Infinite M NANO+ microplate reader (Tecan, Switzerland). UV−vis absorption spectra were recorded using the UV-2550 spectrophotometer (Shimadzu, Japan). TEM images were acquired using the HT-7700 transmission electron microscope (Hitachi, Japan). The size distribution and ζ potential were analyzed with the Zetasizer Nano ZS90 instrument (Malvern, U.K.). FT-IR spectra were obtained using a Nicolet iS50 Fourier transform infrared spectrometer (Thermo Fisher, USA). Emission spectra were recorded with an FLS980 transient fluorescence spectrometer (Edinburgh, UK). Near-infrared (NIR) light source used the 980 nm semiconductor laser (Model: MDL-III-980-2W, Changchun New Industries Optoelectronics Technology Co., Ltd., Changchun, China) was used for in vitro and in vivo NIR irradiation experiments. The UV light source was a 365 nm ultraviolet lamp (Model: ZLUVLAMP001, 10 W). Confocal fluorescence imaging was conducted on a Nikon C2 confocal laser scanning microscope (Nikon, Japan). Flow cytometry analysis was carried out using the FACSCanto II flow cytometer (USA). PAGE characterization was performed on the EPS 300 electrophoresis apparatus (Tanon, Shanghai), and gel images were captured with the UV transilluminator of the UVP GelDoc-It 310 Imaging System.

Synthesis of UCNP.

Upconversion nanoparticles (UCNP) were synthesized using the thermal decomposition method1. Initially, 1 ml of core precursor solution containing 1.59 mmol $YCl_3 \cdot 6H_2O$, 0.40 mmol $YbCl_3 \cdot 6H_2O$, and 0.01 mmol $TmCl_3 \cdot 6H_2O$ was added to a mixture of 15 ml oleic acid and 30 ml octadecene under a nitrogen atmosphere. The solution was heated to 65°C for 20 min to remove methanol, then further heated to 170℃ for 30 min. After cooling to 40°C, 10 ml methanol solution (core precursor: NaOH: NH_4F = 1:2.5:4) was added and stirred for 30 min at 40°C. Subsequently, the temperature was raised to 80°C and then to 150°C successively, with each temperature

being maintained for 20 min to remove methanol and a small amount of water. After complete methanol removal, the temperature was increased to 300°C, and the reaction was carried out for 1 h under nitrogen protection. After cooling to room temperature, ethanol was added, and the product, core $NaYF_4$:Yb/Tm, was obtained by centrifugation and washing. Similarly, 1 ml of shell precursor solution containing 2 mmol $YCl_3 \cdot 6H_2O$ was added to a mixture of 15 ml oleic acid and 30 ml octadecene under nitrogen. The subsequent steps for heating, methanol removal, and washing were performed to obtain shell $NaYF_4$. Finally, 0.2 mmol of core precursor was added to 4.5 ml oleic acid and 12 ml octadecene, and the mixture was heated to 300°C. Shell precursor was added dropwise at a rate of 0.2 ml/10 min using a syringe, followed by a 5 min incubation period. After cooling to room temperature, a small amount of anhydrous ethanol was added, and the product was sonicated, centrifuged, and washed to obtain core-shell upconversion nanoparticles $NaYF_4$:20% Yb, 0.5% Tm@$NaYF_4$ (UCNP).

Assembly of sgAPC strand

The sgAPC complex was prepared by hybridizing sgRNA with photocleavable (PC) strand, thiol-modified (SH-DNA) strand, and APE1-cleavable (AP) strand. The reaction mixture contained 20 μL nuclease-free water, 10 μL 5× RNA annealing buffer, 5 μL 100 μM sgRNA, 5 μL 100 μM PC strand, 5 μL 100 μM SH-DNA, and 6 μL 100 μM AP strand. This mixture was heated to 90°C for 15 min in a PCR thermocycler, followed by gradual cooling to 25°C at a rate of -0.1°C/s to induce hybridization. To prevent thiol group oxidation, 10 μL of TCEP solution was added, and the mixture was incubated for 3 h. Excess TCEP was removed via centrifugation using 3K Amicon ultrafiltration tubes, and the resulting sgAPC complex was stored at 4°C in the dark.

Synthesis of UC@SiO_2-NH_2

First, 0.1 g cetyltrimethylammonium bromide (CTAB) was dissolved in 20 mL deionized water, followed by the addition of 500 μL UCNP dispersion (10 mg/mL) with stirring until clear. Then, 20 mL water, 3 mL ethanol, and 100 μL 2 M NaOH were added, and the mixture was heated to 70°C. TEOS (50–100 μL) was added in increments to control silica shell thickness, with stirring for 1 h after each addition. For

amino functionalization, the as-prepared UC@SiO_2 was dispersed in 5 mL ethanol, mixed with 600 μL (3-aminopropyl)trimethoxysilane (APTES), and stirred at 65°C for 2 h. The product (UC@SiO_2-NH_2) was collected by centrifugation (12,000 rpm, 5 min), washed with ethanol and water, and resuspended in 500 μL PBS (pH 7.4).

Establishment of Tumor Senescence Mouse Mode

UC@SiO_2-NH_2 was conjugated with sgAPC complex via Sulfo-SMCC crosslinking. Briefly, 100 μL UC@SiO_2-NH_2 dispersion in PBS buffer (pH=7.4) was mixed with 1 mL Sulfo-SMCC solution (3 mg Sulfo-SMCC in 1 mL DMF) and incubated for 2 h. The resulting UC@SiO_2-NH_2-Sulfo-SMCC was collected by centrifugation (12,000 rpm, 15 min), washed three times, and resuspended in 1 mL PBS (enzyme-free water). sgAPC complex was added at a molar ratio of 200:1 (relative to UCNPs), and the mixture was incubated at 4°C for 24 h in the dark. Unreacted DNA strands were removed by centrifugation, yielding UC@SiO_2-sgAPC. Finally, Cas9 protein was added at varying molar ratios (0:1 to 200:1 relative to UCNPs), incubated at 4°C for 2 h, and excess Cas9 was removed by centrifugation to obtain UC@SiO_2-RNP (UCRP).

Synthesis of UC@SiO2-RNP/PLL-TPP(UCRP-TPP)

Using Li et al.'s method 2, PLL-TPP was prepared by mixing TPP (0.1 mmol) and EDC (0.2 mmol) in PBS buffer (pH=7.4) for 15 minutes to activate carboxyl groups. Then, NHS solution (0.5 mmol) was added and stirred for another 15 minutes. PLL (5 mg) was added and left to stir overnight at room temperature. After centrifugation, PLL-TPP was obtained in enzyme-free water. Subsequently, 2.5 mg of UCRP was added to PLL-TPP and stirred in the dark at 4°C for 12 hours. After centrifugation, UCRP-TPP was obtained. UCRP-PLL was prepared similarly by mixing 2.5 mg of UCRP with 5 mg of PLL.

CRISPR/Cas9 cleavage experiments based on light and enzyme control in vitro

PAGE Gel Electrophoresis: Using the mitochondrial ND4 gene fragment as a template, a 852-bp dsDNA target sequence was obtained via PCR. A ternary complex consisting of 0.5 μL of 2 μM sg-PC chain, 0.5 μL of 1 μM Cas9 protein, 1 μL of 10×NEB 3.1 buffer, and 8 μL of water was incubated. The mixture was then exposed to UV light (λ= 365 nm, 5.0 mW/cm^2) for varying durations. Subsequently, 0.5 μL of 100 nM

dsDNA target sequence was added, followed by incubation at 37°C for 1 hour. After the reaction, 1 μL of 1 mg/ml proteinase K was added, and the mixture was heated at 65°C for 10 minutes to degrade Cas9 protein and sgRNA. Finally, 2 μL of DNA loading buffer was added, and 2% agarose gel electrophoresis (120 V, 30 minutes) was performed for analysis.

Fluorescence Assay

5 μL of 100 μM TS chain, F-NTS chain, and B-NTS chain were mixed with 10 μL of 5×DNA annealing buffer and 25 μL of enzyme-free water. The mixture was heated in a PCR instrument at 95 °C for 5 minutes, followed by gradual cooling to 25 °C at a rate of -0.1 °C/s to form FQ target chains. UCRP at a concentration of 300 μg/ml was irradiated under near-infrared light (1.2 W/cm^2) for different time. Subsequently, 300 nM FQ target chain and 2 μL of 10×NEB 3.1 buffer were added, and the mixture was incubated at 37 °C for 2 hours. Finally, fluorescence emission spectra were recorded using an FLS980 transient fluorescence spectrometer with an excitation wavelength of 488 nm.

Mitochondrial co-localization analysis

To analyze the localization ability of UCRP-TPP with mitochondria in cells, UCRP-TPP synthesized with carboxyfluorescein (FAM)-labeled sgRNA was used (λex: 494 nm; λem: 522 nm). Hela cells were seeded in confocal dishes (0.5×10^5 cells/well) and cultured for 24 hours. Subsequently, 50 μg/ml of UCRP-TPP or UCRP/PLL was added for co-incubation. For subcellular localization studies, after treatment at different time points (3, 6, 9 hours), 20 nM of mitochondrial tracking probe (Mito-Tracker Red CMXRos, λex: 579 nm; λem: 599 nm) was added and incubated for 10 minutes for mitochondrial staining. After washing three times with PBS, the cells were imaged using a Nikon C2 confocal laser scanning microscope.

Mitochondrial Membrane Potential (MMP) Assay

Changes in mitochondrial membrane potential (Δϕm) were assessed using the JC-1 assay kit (Beyotime) in Hela, MCF-7, and MCF-10A cells. Cells were seeded in confocal dishes (1×10^5 cells/well) and cultured overnight. They were then treated with 60 μg/ml of UCRP-TPP for 9 hours. The control group remained unexposed to near-

infrared (NIR) light , while the experimental groups were subjected to NIR light (980 nm, 1.2 W/cm^2) for 30 minutes (with intermittent irradiation to prevent temperature increase). Following incubation in fresh medium for 48 hours, cells were washed with PBS and stained with the JC-1 working solution at 37°C for 10 minutes. Subsequent imaging was performed (λex: 488 nm, λem: 520 nm; λex: 552 nm, λem: 590 nm).

qPCR analysis of mitochondrial DNA copy number

Genomic DNA from HeLa, MCF-7, and MCF-10A cells was extracted using the TIANamp Genomic DNA Kit (purchased from Beijing Tiangen Biotech Co., Ltd.). β-actin was selected as the reference gene. D-loop was used to detect the relative expression levels of mtDNA. Quantitative polymerase chain reaction (qPCR) analysis was performed using TransStart Green qPCR SuperMix (TransGen). The relative mitochondrial DNA copy number was calculated using the $2^{-\Delta\Delta Ct}$ method. All PCR primers are listed in Table S1.

Western blotting

MCF-7 cells were divided into three groups: the control group, which was cultured in PBS without any other treatment; the -NIR group, which was incubated with 60 μg/ml UCRP-TPP for 9 hours but not subjected to near-infrared (NIR) irradiation; and the +NIR group, which was incubated with 60 μg/ml UCRP-TPP for 9 hours and then exposed to NIR light (980 nm, 1.2 W/cm²) for 30 minutes using an intermittent irradiation method (2 minutes of irradiation followed by a 30-second pause) to prevent temperature elevation. After the above treatments, all groups were cultured in fresh medium for another 48 hours. Total proteins were extracted from the cells using lysis buffer and quantified. Protein samples were denatured in 1× SDS-PAGE protein sample loading buffer at 100°C for 5 minutes, and 25 μg of each protein sample was used for Western blotting analysis. After adjusting to the same concentration, the proteins were separated by SDS-PAGE gradient gel electrophoresis and then transferred to a PVDF membrane. The membrane was blocked with QuickBlock™ Block Buffer (Beyotime, China). Subsequently, the membrane was incubated with primary antibodies against ND4 (1:50, Abcam, Cat# ab116897) and Tubulin (1:1000, e.g., Abcam, cat# ab6046) overnight at 4°C. After washing with TBST, the membrane was incubated with HRP-

coupled secondary antibodies for 1 hour at room temperature. Finally, the protein bands were detected using the Bio Spectrum® 615 imaging System, with Tubulin serving as the internal reference.

Cell Viability Assay

Cell viability was assessed using three methods: (i) Calcein/PI viability assay kit (Beyotime), (ii) CCK-8 assay, and (iii) Annexin V-FITC/PI apoptosis assay kit (YEASEN).

(i) The Calcein/PI viability assay kit was utilized to evaluate cell viability under different conditions. Specifically, HeLa, MCF-7, and MCF-10A cells were seeded in confocal dishes at a density of 1×10^5 cells per well and incubated for 24 hours. After incubation with 60 μg/ml UCRP-TPP for 9 hours, the control group was not subjected to near-infrared (NIR) treatment, while the experimental groups of cells were irradiated under NIR light (980 nm, 1.2 W/cm^2) for 30 minutes (to prevent temperature elevation due to infrared light exposure, an intermittent irradiation method was adopted, with irradiation for 2 minutes followed by a 30-second pause). The cells were then washed with fresh culture medium and incubated for an additional 48 hours. Subsequently, cells were washed with PBS and stained with Calcein AM (λex: 488 nm; λem: 520 nm) and PI (λex: 552 nm; λem: 580 nm) according to the instructions provided with the kit, followed by imaging under confocal microscopy.

(ii) Cell viability was also assessed using the CCK-8 method. Cells (5×10^3 cells/well, 100 μL) were seeded in 96-well plates and cultured for 24 hours. The same procedures described above were repeated, and cell viability was measured using the Cell Counting Kit-8 (CCK-8). Results were obtained using an enzyme-linked immunosorbent assay (ELISA) reader.

(iii) Annexin V-FITC/PI apoptosis assay kit was employed to detect cell viability. Cells were seeded in 6-well plates (1×10^5 cells/well), and the same experiments as described above were repeated. Results were obtained using a flow cytometer.

Animal Experiment

Six- to eight-week-old female nude mice (BALB/c strain) were purchased from the East China Normal University Experimental Animal Center in Shanghai, China. All mouse

experiments were conducted according to the protocol approved by the Animal Experiment Ethics Committee of East China Normal University (No. m20240204). The xenograft tumor model was established by inoculating 1×10^6 MCF-7 cells (in 100 μL) into the dorsal hind limb area of the mice to simulate a tumor growth environment. A total of 20 mice were used in this study, randomly divided into five groups to evaluate the effects of different treatments on tumor growth. When the tumor volume reached 100 mm^3, on day 1, the mice in each group received injections of UCRP-PLL, UCRP-TPP, UCNP-TPP, or PBS as a control. This was followed by the first treatment session, during which NIR light (980 nm, 1.2 W/cm^2) was applied intermittently for 30 minutes over the next two days. To prevent local temperature elevation (which might cause off-target tissue damage), an intermittent irradiation protocol was adopted: 2 minutes of continuous NIR exposure followed by a 30-second pause, repeated cyclically until the total 30-minute irradiation duration was achieved. We also assessed the effects of UCRP-TPP treatment without near-infrared light to gain a comprehensive understanding of its therapeutic potential. The treatment was repeated every three days for a total of three sessions, with body weight and tumor volume recorded throughout to monitor the therapeutic effect. Tumor volume was calculated using the formula $V = a\times b^2$, where "a: tumor length ; b: tumor width. Mice were euthanized after the final treatment, and tumors were collected for analysis.

Table S1. Sequences of the oligonucleotides used in our work

名称	序列（5'-3'）
sgRNA	GGTAATGATGTCGGGGTTGAGTTTTAGAGCTAGAAATAGCAAG TTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACC GAGTCGGTGCTTT
B-NTS	AAAATAGGGTTAGGGTTAGGG TTA-BHQ1
F-NTS	FAM-GGGAGTCATCAACACAGCTCAGTTCGTAAA
TS	TTTACGAACTGAGCTGTGTTGATGACTCCCTAACCCTAACCCT AACCCTA TTTT
PC strand	ACCCCGACAT/iPCLink/CATTACCTGTAACCTGTCTCTCTGC
SH-DNA	SH-TTTTTTTGCAGAGAGACAGGTTACA
AP strand	CGACTCCACTTTTTCA/idSp/AGTTGATAACG/idSp/ACTAGCCTT GCTGAG
$mtND_4$-F	CACATAGCCTACCCCTTCCT
$mtND_4$-R	AAGTCAGGGTTAGGGTGGTT
β-actin-F	GGATGCAGAAGGAGATCACTG
β-actin-R	CAAGTACTCCGTGTGGATCG
$Mt_{D\text{-}loop}$ -qPCR-F	CACCAGCCTAACCAGATTTC
$Mt_{D\text{-}loop}$ -qPCR-R	GGGTTGTATTGATGAGATTAGT

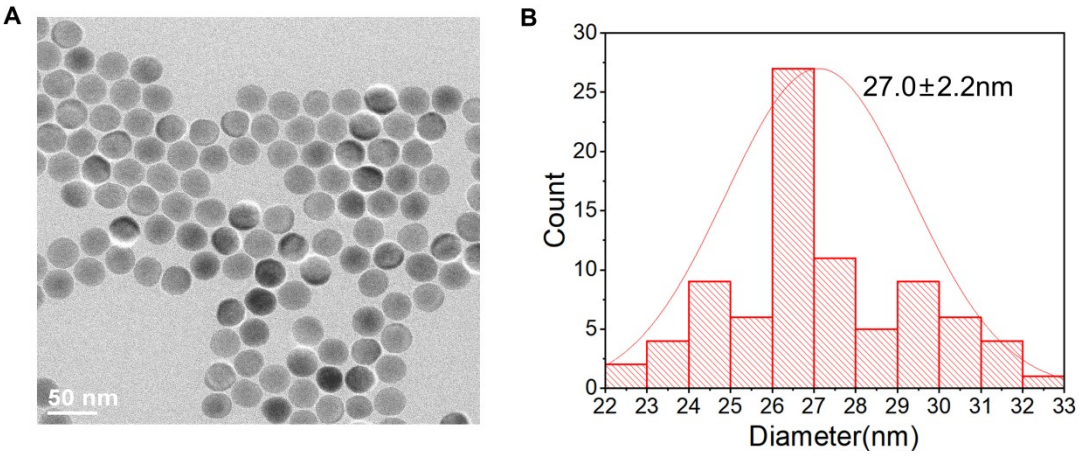

Figure S1. (A) TEM image of UCNPs (scale bar: 50 nm) and (B) corresponding particle size distribution in (A).

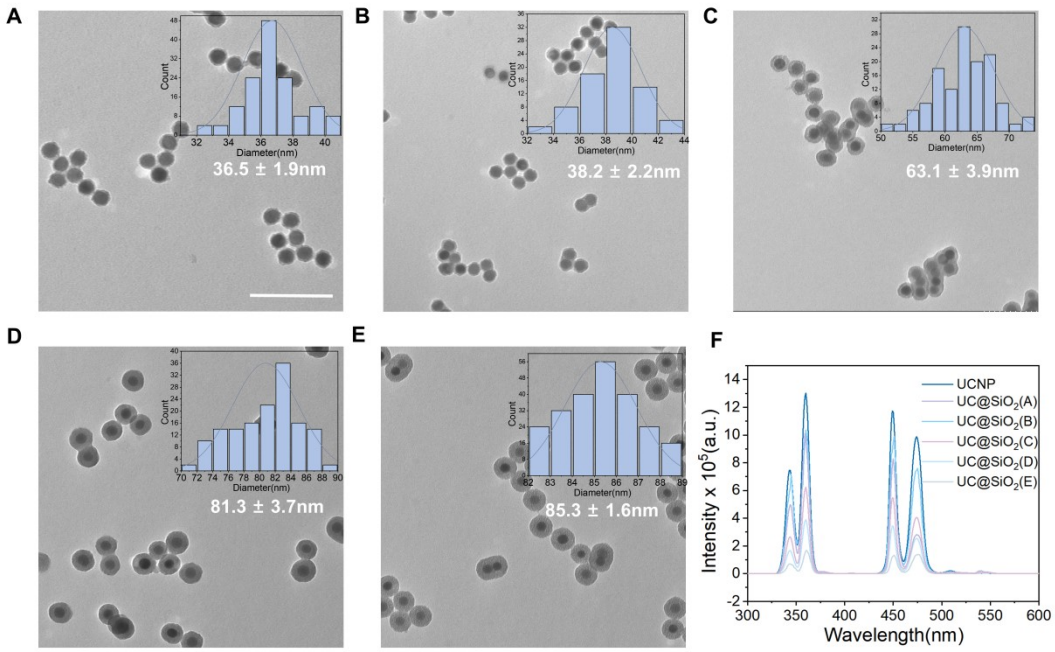

Figure S2. The particle size of UCNP@SiO_2 with different shell thicknesses, (a) 36.5±1.9 nm, (b) 38.2±2.2 nm, (c) 63.1±3.9 nm, (d) 81.3±3.7 nm, (e) 85.3±1.6 nm, and (F) corresponding fluorescence emission spectra excited at 980 nm. (scale bar: 50 nm)

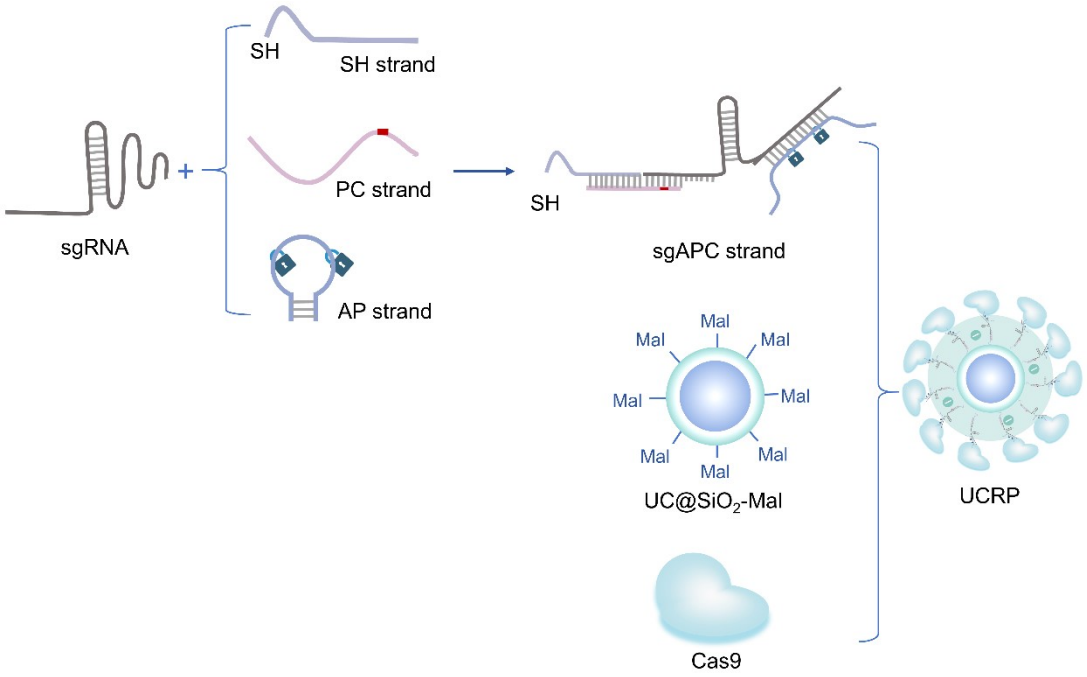

Figure S3. The scheme for the design of UC@SiO_2–sgAPC/Cas9 (UCRP) delivery system.

The PC strand, SH strand, AP strand, and sgRNA strand hybridize to form the sgAPC complex, which is covalently linked to UC@SiO2-Mal via the thiol group on SH strand and the maleimide group on UC@SiO2-Mal. Cas9 is then loaded onto the complex through electrostatic and recognition interactions with the positively charged nucleic acids.

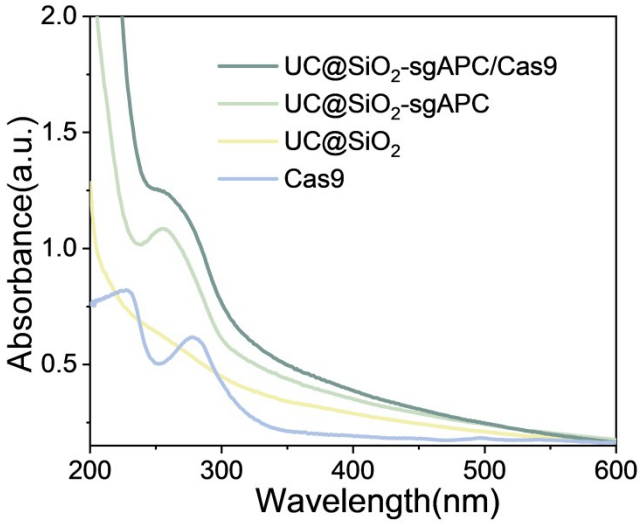

Figure S4. UV-visible absorption spectra of Cas9, UC@SiO_2, UC@SiO_2-sgAPC, and UC@SiO_2-sgAPC/Cas9 (UCRP).

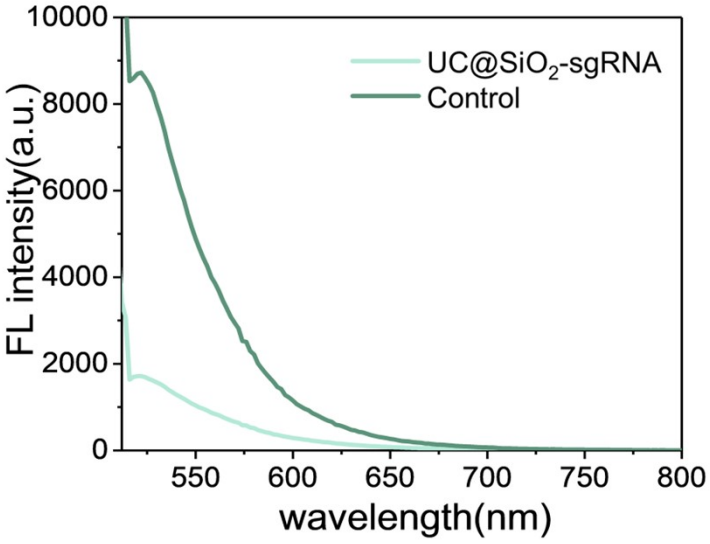

Figure S5. Fluorescence intensity of the supernatant was measured before and after the synthesis of UC@SiO_2-sgRNA. The control group represents the fluorescence intensity of the supernatant containing FAM-labeled sgRNA without incubation with UCNPs.

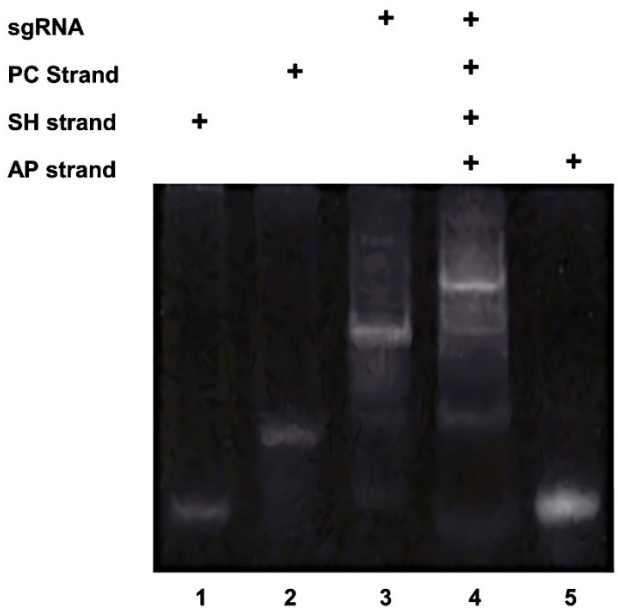

Figure S6. Gel electrophoresis image of SH strand, PC strand, sgRNA, AP strand, and their hybridization products of APC-sgRNA. Lane1: SH strand; Lane2: PC strand; Lane3: sgRNA; lane 4: APC-sgRNA lane 5: AP strand.

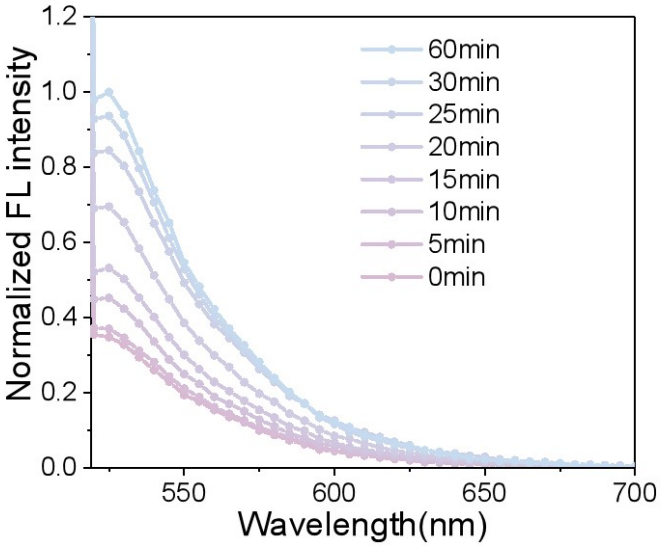

Figure S7. Fluorescence intensity of target DNA cleaved by UCRP upon NIR light exposure.

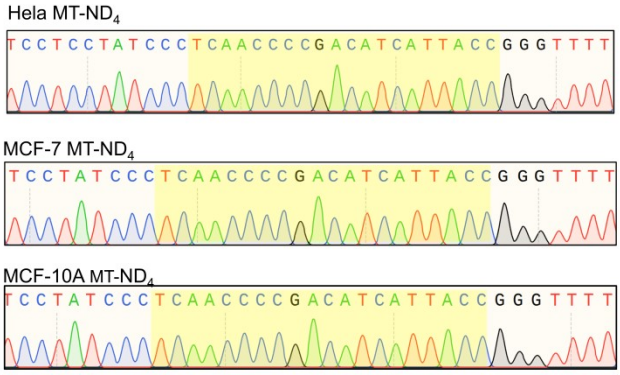

Figure S8. Genetic sequencing analysis of Mitochondria DNA (mtDNA) in HeLa, MCF-7, and MCF-10A Cells.

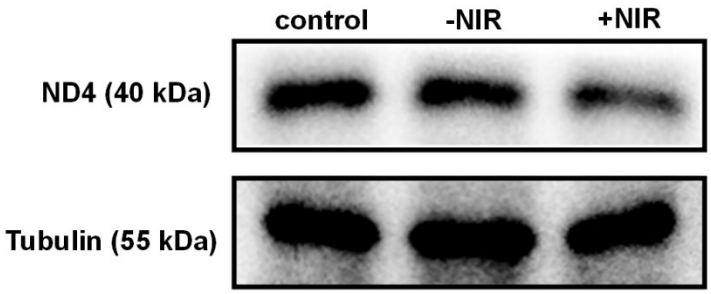

Figure S9. Representative Western blot images of ND4 and Tubulin protein expression in MCF-7 cells after different treatments.

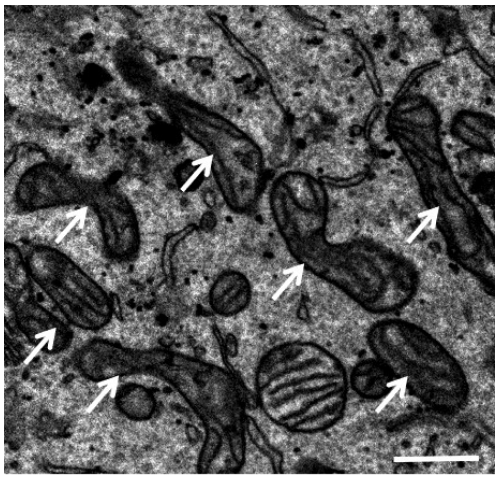

Figure S10. Bio-TEM image of mitochondria in MCF-7 cells treated with UCRP-TPP without NIR light illumination.

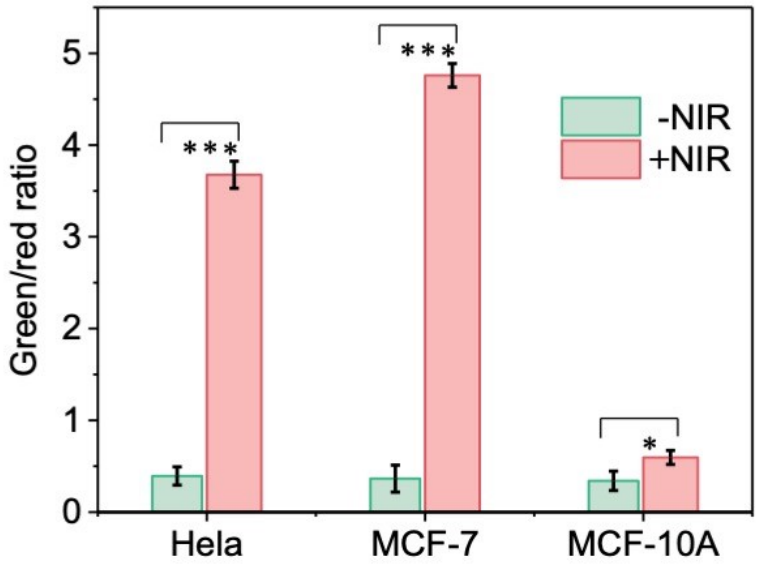

Figure S11. Ratio of green/red fluorescence intensity shown in Figure 5(F). *P<0.1, ***P<0.001. Data are presented as the mean ± SD (n = 3).

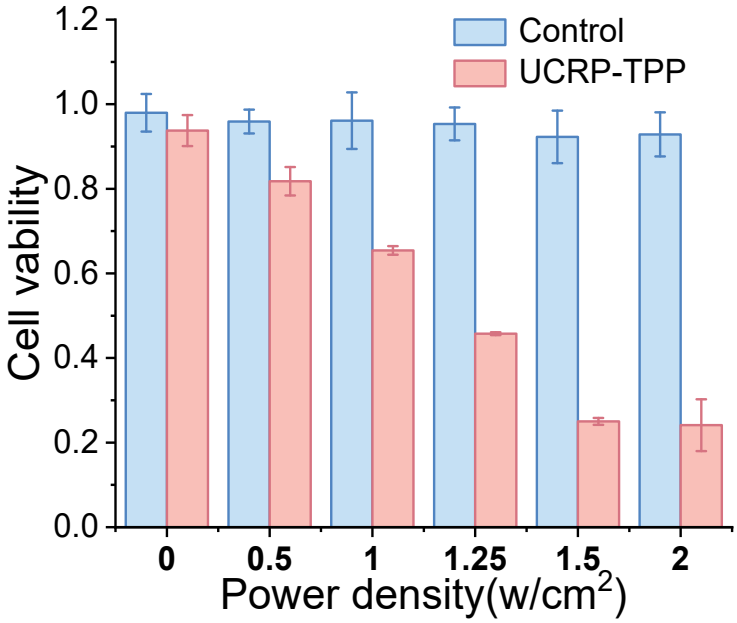

Figure S12. CCK-8 analysis of Hela cells treated with different concentrations of UCRP-TPP.