Supporting Information

Cell-type-specific CRISPRization of mitochondrial DNA using bifunctional biodegradable silica nanoparticles

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Contents

General Experimental Information

All chemicals were purchased from commercial suppliers and used without further purification. All aqueous solutions were prepared by using ultrapure water from a Milli-Q system. Tetraethyl orthosilicate (TEOS) and (3-aminopropyl)triethoxysilane (APTES) were purchased from Shanghai Titan Scientific Co., Ltd. Bis[3(triethoxysilyl)propyl]-disulfide (BTEPDS) were obtained from Sarn Chemical Technology Co., Ltd. Hyaluronic acid (HA) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. All the fluorescent organelle stains were purchased from Invitrogen. Fluorescein isothiocyanate (FITC) was purchased from Adamas. Bovine serum albumin (BSA) was obtained from Kehbio. Antibodies used for western blotting (WB) were obtained from the following vendors: MTCO1 mAb (#ab203912, Abcam), MTCO2 polyAb (#55070-1-AP, Proteintech), GAPDH (HRP-60004, Proteintech). Cas9-His was expressed and purified using plasmid (cat: 62934, Addgene). The sgRNAs (The COX1 sgRNA sequence is as following: GGCCCAGCUCGGCUCGAAUAUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU. The COX2 sgRNA sequence: UAUGAGGGCGUGAUCAUGAAUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU) were designed according to the literature[1] and purchased from Tsingke Biotechnology. Zeta potential and polydispersity of nanoparticles were recorded using a Malvern Nano-ZS90. Transmission electron microscopy (TEM) images were obtained by HT7700 (HITACHI, Japan). Western blot and fluorescent image were captured using Invitrogen iBright 1500. Confocal laser scanning microscopy (CLSM) images of cells were taken on Leica DMi8. Flow Cytometry experiments were carried out on a BD FACSMelody™.

Synthesis of Materials[2] and Performance Test

Synthesis of Protein@BSNP

Triton-100 (0.36 mL), n-hexanol (0.36 mL), and cyclohexane (1.5 mL) were pre-stirred continuously at room temperature. Then 60 μL of 1 mg/mL protein mixed with TEOS (8 μL) and BTEPDS (16 μL) were slowly added dropwise to the above solution. Finally, ammonia (12 μL) was added. The overall mixed solution was stirred at 350 rpm for 12 h under room temperature. The resulting nanoparticles were precipitated by addition of 4 mL acetone, followed by centrifugation (15000 g, 8 min, 4°C) and further washings with ethanol and water twice, respectively. The obtained Protein@BSNP was weighted and stored at 4°C until use.

Synthesis of N-Protein@BSNP

1 mg Protein@BSNP was dispersed in 200 μL dry ethanol, and 5 μL APTES was added dropwisely. The above reaction solution was stirred at room temperature for 8 h. Then, the nanoparticles were collected by centrifugation (15000 g, 10 min, 4°C), and washed twice with ethanol and water, respectively. The obtained NH2-Protein@BSNP (N-Protein@BSNP) was stored at 4°C until use.

Synthesis of T-Protein@BSNP

1 mg N-Protein@BSNP was dispersed in 200 μL of ethanol, and 10 μL TPP-COOH (100 mM), 10 μL NHS (100 mM), 10 μL EDC (100 mM) together with 2% DIEA were added. After incubation for 24 h at room temperature, the as-prepared TPP-Protein@BSNP (T-Protein@BSNP) was collected by centrifugation (15000 g, 15 min, 4°C), and washed twice with ethanol and water respectively. The obtained nanoparticles were stored at 4°C until use.

Synthesis of H-T-Protein@BSNP

1 mg T-Protein@BSNP was dispersed in 200 μL of water，then 100 μL HA stock solution (1 mg/mL) was mixed. Subsequently, the above solution was stirred for 4 h under room temperature. Finally, the nanoparticles were collected by centrifugation (15000 g, 10 min, 4°C) and washed twice with water. The obtained HA-TPP-Protein@BSNP (H-T-Protein@BSNP) was stored at 4°C until use.

Synthesis of Cas9^{sgRNA}@BSNP and H-T-Cas9^{sgRNA}@BSNP

Cas9/sgRNA complex was freshly prepared with appropriate adjustments according to the literature.[3] Cas9/sgRNA complex was prepared by mixing Cas9 and sgRNA in 10 mM PBS buffer in a molar ratio of 1:1 for 20 min at room temperature. Then Cas9^{sgRNA}@BSNP (CC@BSNP) and HA-TPP-Cas9^{sgRNA}@BSNP (H-T-CC@BSNP) were synthesized according to the same procedure.

Protein labeling
20 mg of BSA was dissolved in 4 mL of $NaHCO_3$ (0.1 M, pH=8.5) to a final concentration of 5 mg/mL. Then 20 μL of FITC (50 mM) was added dropwise to the BSA solution, and the mixture was reacted at 4°C for 2 h. Finally, the labeled BSA (FBSA) was dialyzed three times in 10 mM PBS buffer (pH=7.0) with a cellulose membrane (MWCO 14 kDa, Biosharp).

Protein encapsulation efficiency
Encapsulation efficiency (EE%)：%protein successfully encapsulated in nanoparticles.
The formula is as follows:

EE%=100*[Total protein added-free (unencapsulated) protein]/Total protein added

We chosed FBSA as the model protein to calculate the protein encapsulation efficiency. After the FBSA@BSNP was synthesized, the supernatant was collected by centrifugation, then the fluorescence intensity of supernatant and initial FBSA solution were measured by BioTek (excitation/emission wavelength: 488 nm/520 nm). Based on the fluorescence intensity, the formula became:

EE%=100*[Fluorescence intensity of initial added protein-Fluorescence intensity of free (in supernatant) protein]/Fluorescence intensity of initial added protein

According to the fluorescence intensity recorded, EE% was determined to be 51.2% for FBSA@BSNP.

Stability test
H-T-FBSA@BSNP was incubated with different solvents (PBS, ethanol, acetone, and water) and fetal bovine serum (FBS, 10%) for 48 h and 72 h at 4℃. After that, the stability of the samples was characterized by DLS and TEM.

Nanoparticles biodegradation and protein release experiments
FBSA@BSNP or eGFP@BSNP (0.1 mg/mL) was incubated with GSH (10 mM) for various durations. After that, the resulting supernatant was collected by centrifugation (14000 g, 4℃, 8 min) to measure the fluorescence intensity, and the morphology change was also observed by TEM. FBSA@BSNP or eGFP@BSNP incubated with PBS buffer was used as control.

Biology Experiments

Cell culture
HeLa, A549, HepG2, LO-2, and BEAS-2B were cultured in high-glucose Dulbecco's modified eagle medium (DMEM) containing 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified environment with 5% CO_2 atmosphere.

Cell selective uptake imaging assay
We chosed HeLa as the cell line with high CD44 expression and LO-2 as the negative control to verify the cell-type-specific uptake of H-T-FBSA@BSNP. The two cell lines were seeded separately in 4-well glass bottom dishes and grown for 12 h until 60%~80% cell density. After removing medium, cells were incubated with 50 μg/mL and 100 μg/mL of H-T-FBSA@BSNP for 24 h, respectively. After washing with PBS, the cells were imaged by CLSM (FITC: λex = 488 nm, λem = 520 nm. Cy5: λex = 638 nm, λem = 650-700 nm).

Flow cytometry assay (FACS)
Cells were inoculated in 12-well plates (3×10^5 cells/well) and incubated with 50 μg/mL of different modified FBSA-loaded nanoparticles for 24 h to quantify the cell uptake. Cells were separated from the plates by removing the medium and then treated with 500 μL trypsin at 37°C for 2 min. The detached cells were collected by centrifugation (1000 g, 3 min), followed by being washed three times and suspended with PBS. Fluorescence intensities were analyzed using BD FACSMelody™.

Mitochondrial co-localization imaging assay
HeLa、A549、HepG2 cell lines were seeded separately in 4-well glass bottom dishes and grown overnight. After medium removal, cells were incubated with 50 μg/mL of different modified FBSA-loaded nanoparticles for 24 h. Then, the three cells were further co-stained with MitoTracker (18 nM, MitoTracker® Deep Red, M7512) in the growth medium for 1 h at 37°C. After washing with PBS twice,

the cells were imaged by CLSM (FITC: λex = 488 nm, λem = 520 nm. Cy5: λex = 638 nm, λem = 650-700 nm).

Bio-TEM assay

HeLa cell line was inoculated in 10 cm cell culture dishes and incubated with H-T-FBSA@BSNP for 24 h (total number of cells was 10^6). Cells were separated from the dishes by removing the medium and then treated with 3 mL trypsin at 37°C for 2 min. The cells were collected by centrifugation and washed three times with PBS (1000 g, 3 min). Next, 2.5% glutaraldehyde solution was added (the fixative was filled to the top of the centrifuge tube so that the sample was completely submerged in the fixative) and placed at 4°C for 12 h. The cell embedded samples were sectioned by using a Leica Ultracut UCT instrument. The bio-TEM images were acquired by viewing the lead citrate stained ultra-thin section on a JEOL JEM-1220 TEM (100 kV) with a tungsten electron source.

Cell toxicity assay

HeLa or LO-2 cells (10^4 cells) were seeded into 96-well plates and cultured in 200 μL medium for 24 h at 37°C. Cells were then incubated with the given concentrations (0, 50, 100, 150, 200 μg/mL) of different nanoparticles in 200 μL of medium for 24 h. After incubation, cells were mixed with cell culture medium containing CCK-8 reagent (ApexBio Technology, 10 mg/mL) for 1 h at 37°C. After shaking for 20 min, absorbance at 460 nm was measured with BioTek.

Western blot assay (WB)

The mtDNA editing was evaluated by WB as described in detail below. HeLa cells grown in 12-well plates for 24 h (3×10^5 cells/well) were treated with different concentrations of H-T-CC@BSNP (50 μg/mL, 200 μg/mL) for different time periods (12 h, 24 h, 48 h). The cells were then washed twice with PBS and lysed in PBS lysis buffer (containing 0.1% Triton-X and 100 μM PMSF, 80 μL per well). Next, 20 μL of 5×SDS loading buffer was added to the cell lysate and heated at 95°C for 10 min to denature the proteins. The prepared samples were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (4°C, 30 V for 8 h), and then the membranes were blocked with 5% milk/TBST for 1.5 h at room temperature. Primary antibodies (COX1, 1:2000 dilution; COX2, 1:5000 dilution) and HRP-conjugated mouse anti-GAPDH (1:7500 dilution) were incubated overnight at 4°C. Then HRP-conjugated goat anti-rabbit IgG (1:7500 dilution) was added and further incubated for 1 h at room temperature. The blots were recorded with Invitrogen iBright 1500.

Real-time quantitative PCR analysis (qPCR)

qPCR was performed for quantitating the mtDNA level. HeLa cells were incubated with H-T-CC@BSNP (50 μg/mL, 200 μg/mL) for 24 h, 48 h, followed by washing with PBS and RNA isolation using TRIzol® reagent (Sangon Biotech). The corresponding cDNA was obtained by reverse transcription of the extracted RNA using 5×Prime Script RT Master Mix (TaKaRa). Samples were processed under a heating cycle (37℃, 15 min; 85℃, 5 min; 4℃, ∞). Subsequently, the cDNA templates, the corresponding primers, and 2×S6 Universal SYBR qPCR Mix (NovaBio) were mixed. The PCR program was set with an initial denaturation step at 95°C for 30 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Finally, expression of the endogenous target gene was calculated by using the $2^{-\Delta CT}$ method ($\Delta Ct = Ct_{target}\text{-}Ct_{blank}$).

Next-generation sequencing (NGS)

HeLa cells grown in 12-well plates were incubated with H-T-CC@BSNP (100 μg/mL and 200 μg/mL) for 48 h followed by washing with PBS. Then the whole cell DNA was extracted using Trelief® Hi-Pure Animal Genomic DNA Kit (Tsingke Biotechnology) according to the manufacturer's instructions. Proper primers (Table S1) were used to amplify *COX1* and *COX2* by PCR as described above. The crude PCR products were subjected to NGS.

Tables and Figures

Table S1. Primers used in this study.

Name	Sequence
COX1-F	CGCCGACCGTTGACTATTCT
COX1-R	GGGGGCACCGATTATTAGGG
COX2-F	ACATGCAGCGCAAGTAGGTC
COX2-R	GGGCATACAGGACTAGGAAGC
GAPDH-F	GATGCCCCCATGTTCGTCAT
GAPDH-R	CAAAGAAAGAGGGAGCGGGG
COX1-NGS-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CGCCGACCGTTGACTATTCT
COX1-NGS-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGGGGCACCGATTATTAGGG
COX2-NGS-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CATGCAGCGCAAGTAGGTCT
COX2-NGS-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GAGGGATCGTTGACCTCGTC

Table S2. *COX1* and *COX2* genomic on-target indel sequences. "ref" refers to the human genome reference sequence for each site. Insertions and deletions are shown in red letters or red dashes, respectively.

	mt*COX1* .103_122	H-T-CC1@BSNP (100 μg/mL)			H-T-CC1@BSNP (200 μg/mL)		
	Sequence	Total reads	Insertions	Deletions	Total reads	Insertions	Deletions
ref	TATTTGAGCCGAGCTGGGCC	733	0	0	866	0	0
	-ATTTGAGCCGAGCTGGGCC	0	0	0	0	0	0
	T-TTTGAGGCGAGCTGGGCC	0	0	0	1	0	1
	TATT-GAGCCGAGCTGGGCC	0	0	0	0	0	0
	TATTCGAGCCGAGCT-GGCC	1	0	1	0	0	0
	TATTCGAGCCGAGCTGGG-C	0	0	0	2	0	1
	Indel frequency	1 (0.136%)			3 (0.346%)		

	mt*COX2* .63_82	H-T-CC2@BSNP (100 μg/mL)			H-T-CC2@BSNP (200 μg/mL)		
	Sequence	Total reads	Insertions	Deletions	Total reads	Insertions	Deletions
ref	TCATGATCACGCCCTCATAA	1326	0	0	1126	0	0
	-CATGATCACGCCCTCATAA	0	0	0	1	0	1
	TCATGATCACG-CCTCATAA	1	0	1	1	0	1
	TCATGATCACGCCC-CATAA	1	0	1	0	0	0
	TCATGATCACGCCCTC-TAA	1	0	1	0	0	0
	TCATGATCACGCCCTCAT-A	1	0	1	1	0	1
	TCATGATCACGCCCTCATA-	0	0	0	1	0	1
	Indel frequency	4 (0.302%)			4 (0.355%)		

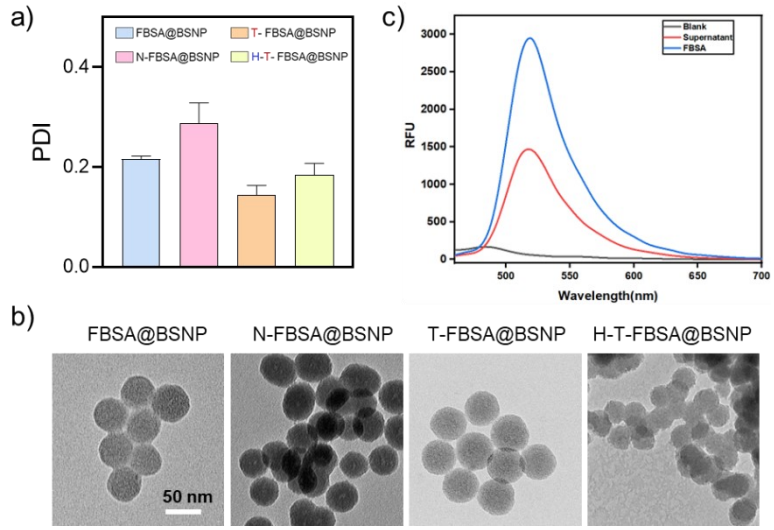

Figure S1. a) PDI data and b) TEM images of FBSA@BSNP, N-FBSA@BSNP, T-FBSA@BSNP, H-T-FBSA@BSNP (scale bar = 50 nm). c) Confirmation of protein encapsulation in BSNP.

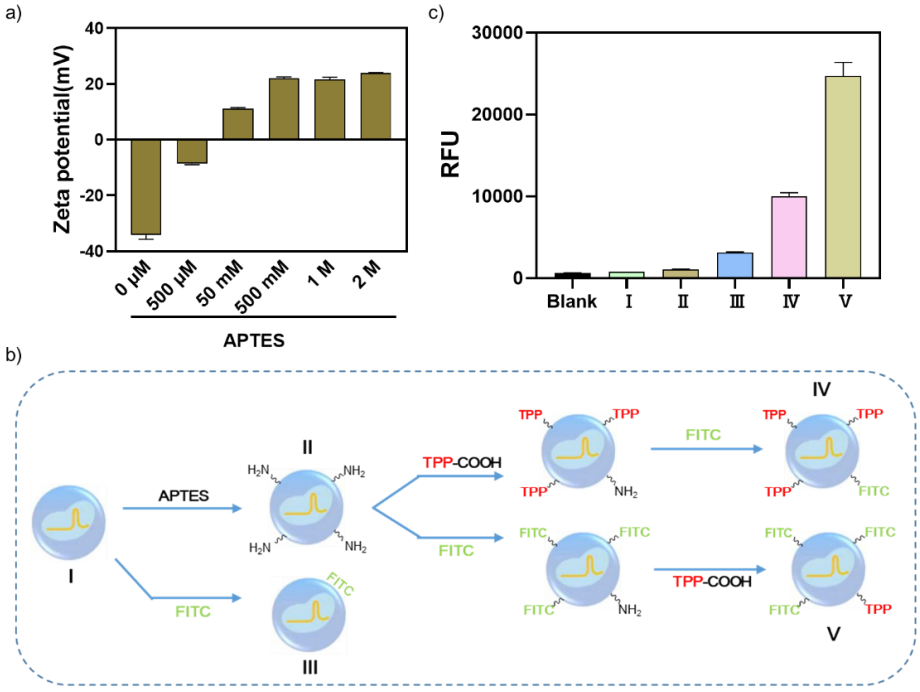

Figure S2. Successful modification verification of -NH_2 and TPP. a) Zeta potentials of FBSA@BSNP before and after modification of different APTES concentrations. b) Schematic diagram of competitive experiment for confirming TPP modification. c) Fluorescence intensity of different nanoparticles.

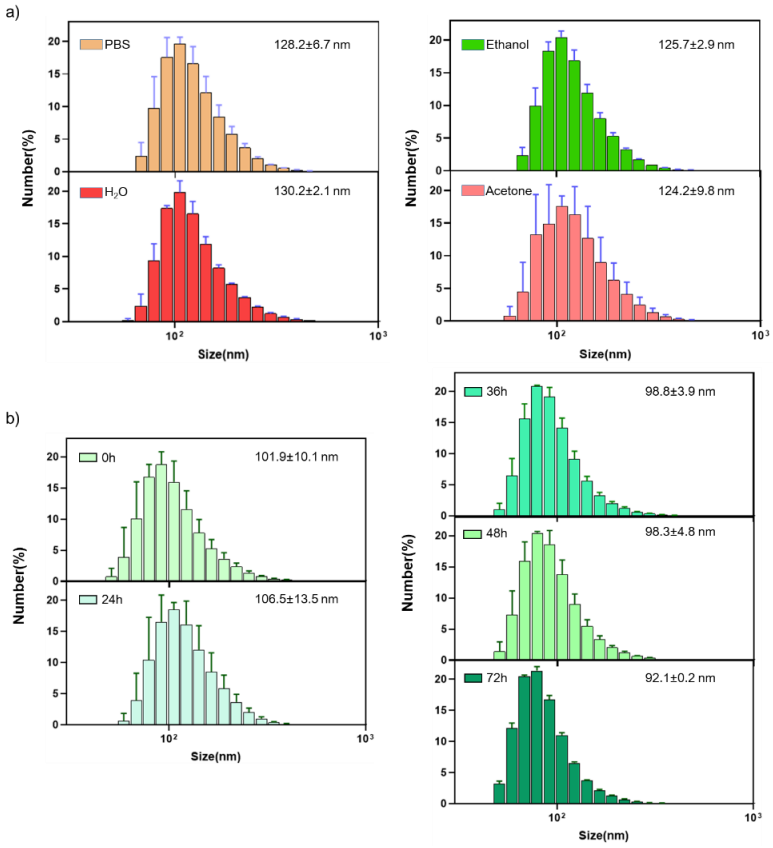

Figure S3. Size distributions of FBSA@BSNP a) in different solvents at 24 h and b) in PBS at 0 h, 24 h, 36 h, 48 h, 72 h.

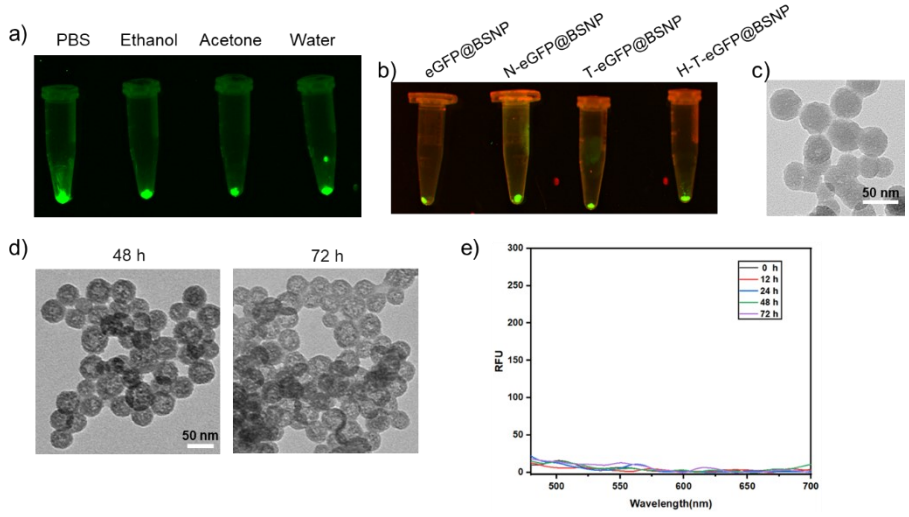

Figure S4. Biological applicability validation of BSNP. a) Fluorescence of eGFP@BSNP after 24 h storage in different solvents. b) Fluorescence of eGFP@BSNP after modification with different groups. c) TEM image of eGFP@BSNP (scale bar = 50 nm). d) TEM images of H-T-FBSA@BSNP dispersed in 10% FBS for 48 h, 72 h. e) Fluorescence of H-T-FBSA@BSNP supernatant after incubation with 10% FBS for different time.

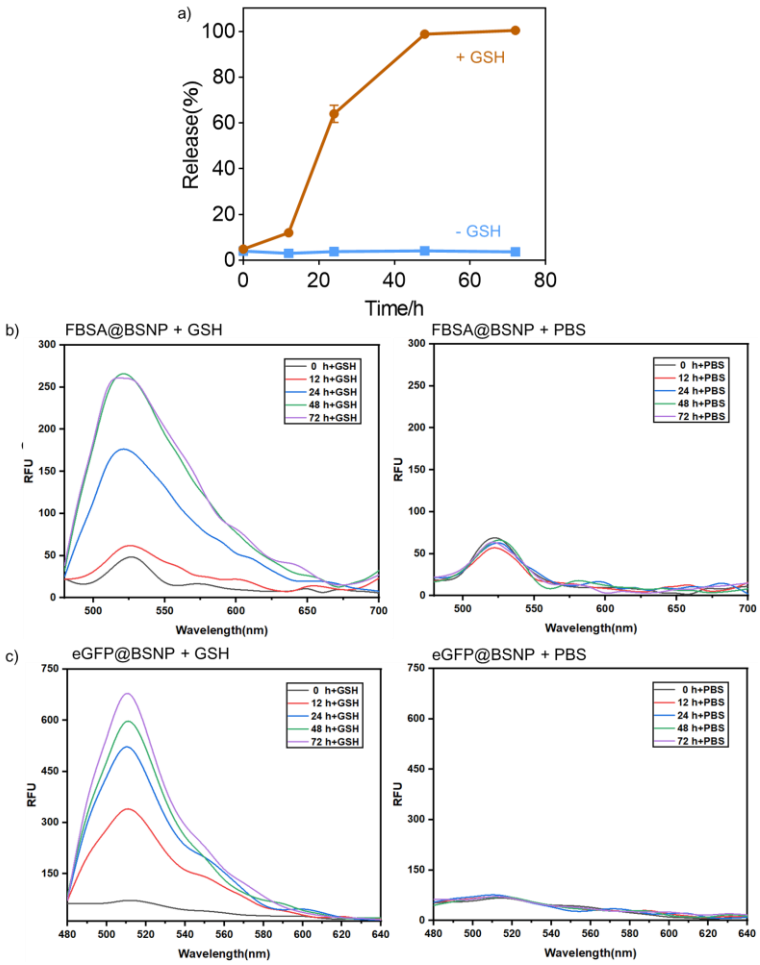

Figure S5. GSH-responsive release profile. a) Accumulated release profiles of FBSA from FBSA@BSNP in the presence of GSH (10 mM). Fluorescence of b) FBSA@BSNP and c) eGFP@BSNP (0.1 mg/mL in PBS buffer) supernatant after incubation with or without 10 mM GSH for different periods of incubation time.

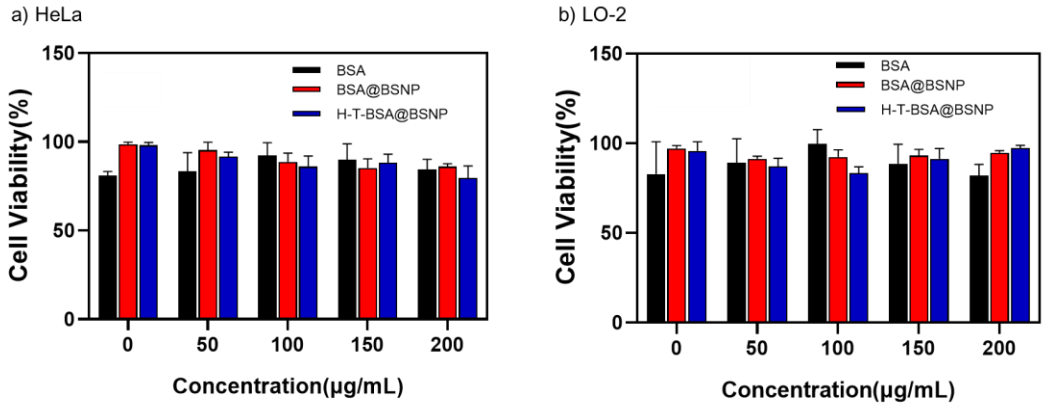

Figure S6. Cell toxicity analysis of a) HeLa and b) LO-2 cells with different concentrations of BSA-loaded nanoparticles for 24 h.

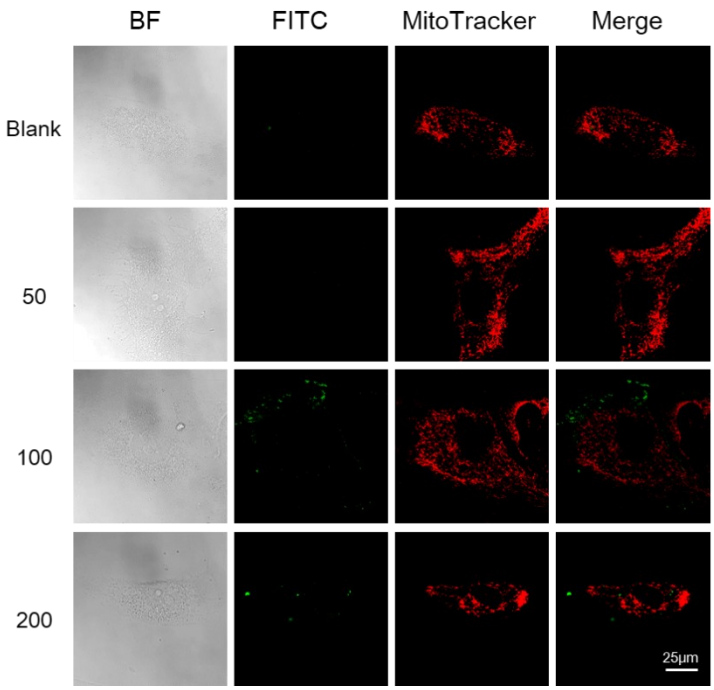

Figure S7. CLSM images of BEAS-2B cells incubated with different concentrations (50-200 μg/mL) of H-T-FBSA@BSNP. FITC (green) channel: FBSA; Cy5 (red) channel: Mito tracker (scale bar=25 μm).

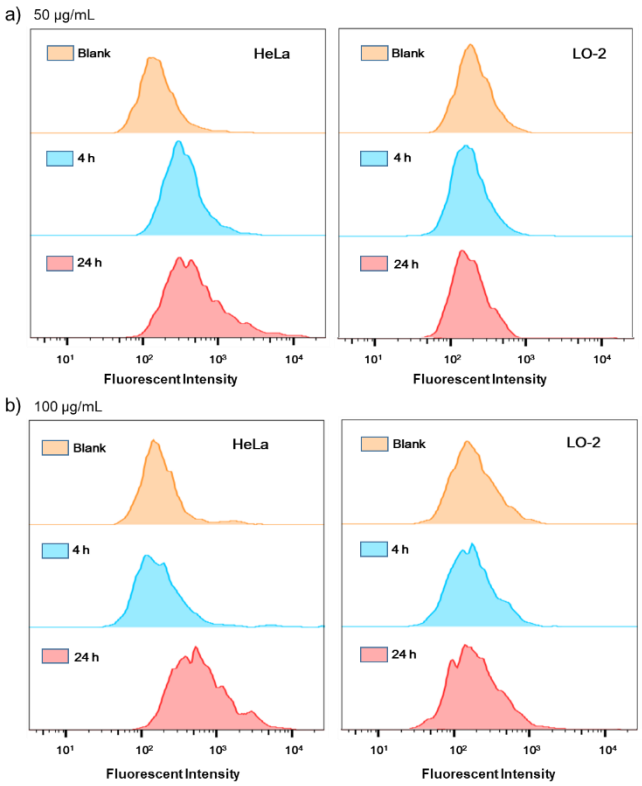

Figure S8. FACS of a) 50 μg/mL and b) 100 μg/mL of H-T-FBSA@BSNP in HeLa and LO-2 cells for 4 h and 24 h.

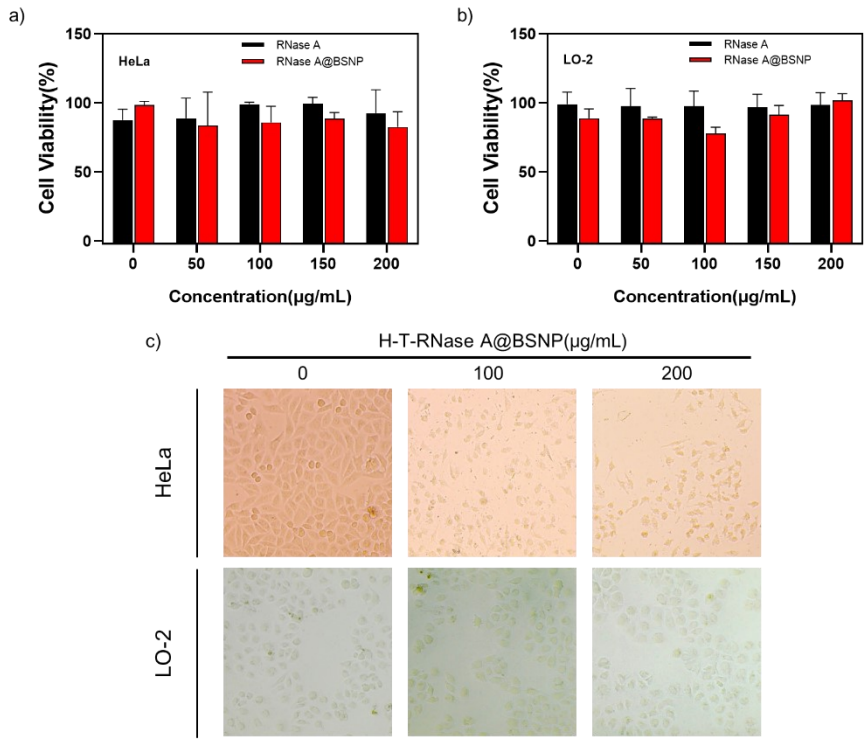

Figure S9. Cell viability analysis of a) HeLa and b) LO-2 cells treated with different concentrations of RNaseA@BSNP for 24 h. c) Images of HeLa and LO-2 cell morphology after incubation with different concentrations of H-T-RNaseA@BSNP for 24 h, respectively.

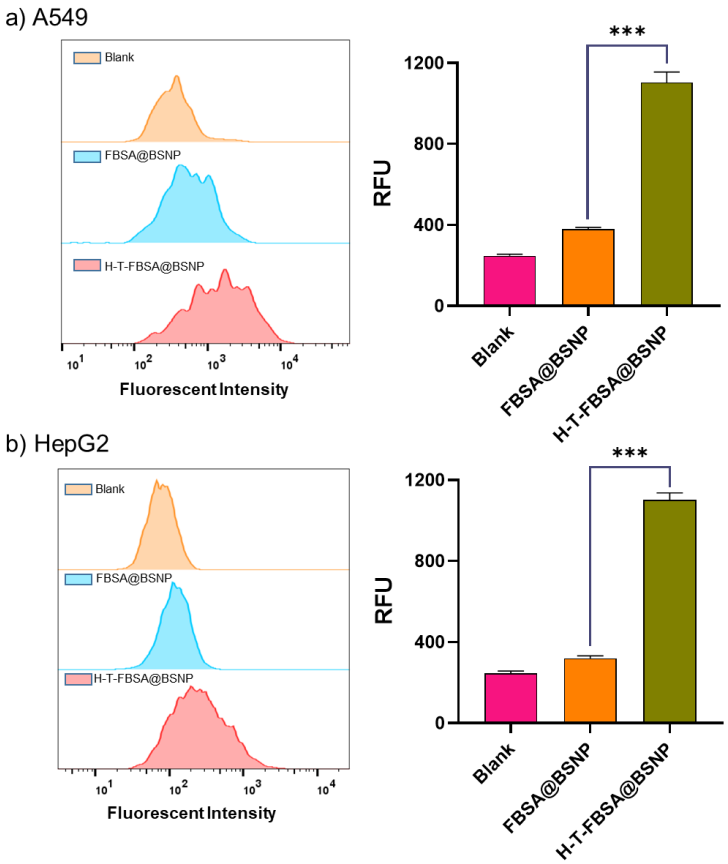

Figure S10. FACS of FBSA@BSNP and H-T-FBSA@BSNP in a) A549 and b) HepG2 cells. *p<0.05, ***p<0.001.

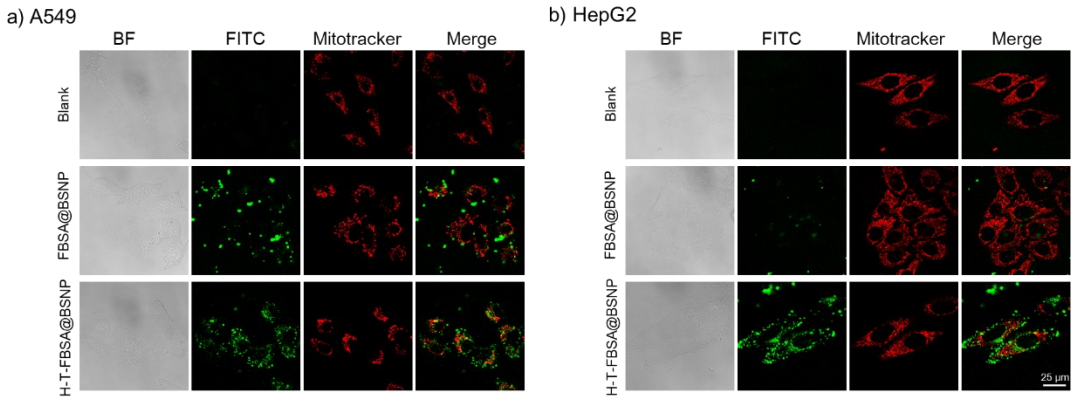

Figure S11. Mitochondrial localization in A549 and HepG2 cells. CLSM images of FBSA@BSNP and H-T-FBSA@BSNP (50 μg/mL) treated a) A549 and b) HepG2 cells for 24 h. FITC (green) channel: FBSA; Cy5 (red) channel: Mito tracker (scale bar = 25 μm).

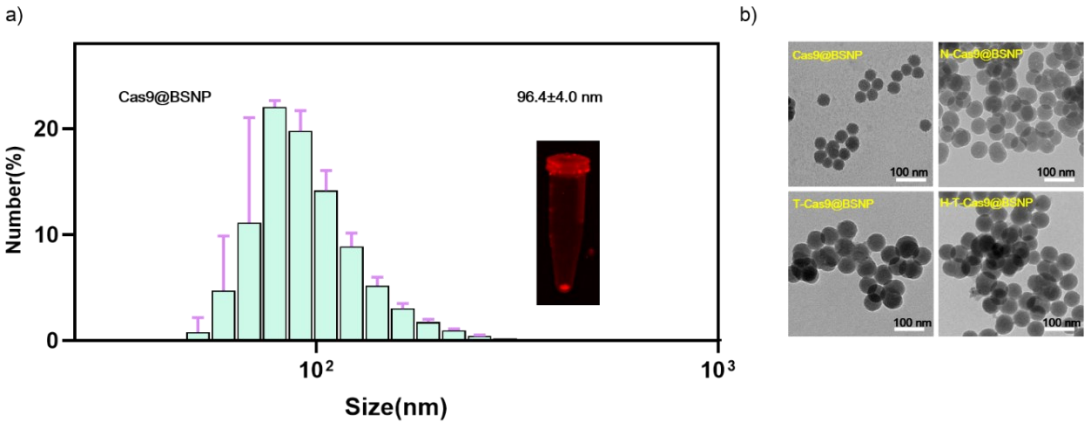

Figure S12. a) Size comparison of BSA@BSNP and Cas9@BSNP. b) TEM images of Cas9@BSNP, N-Cas9@BSNP, T-Cas9@BSNP, H-T-Cas9@BSNP (scale bar=100 nm).

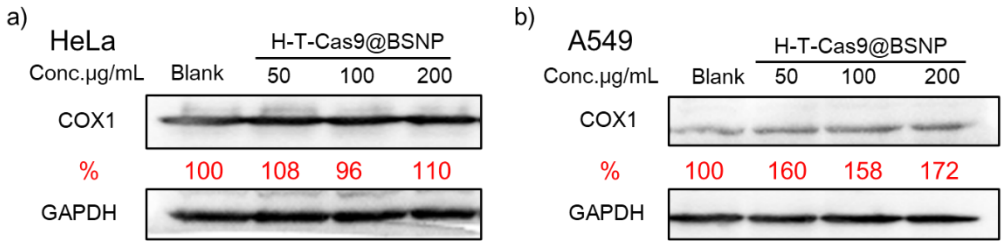

Figure S13. WB analysis of mitochondrial respiratory chain protein (COX1) in a) HeLa and b) A549 cells treated with different concentrations of H-T-Cas9@BSNP for 48 h.

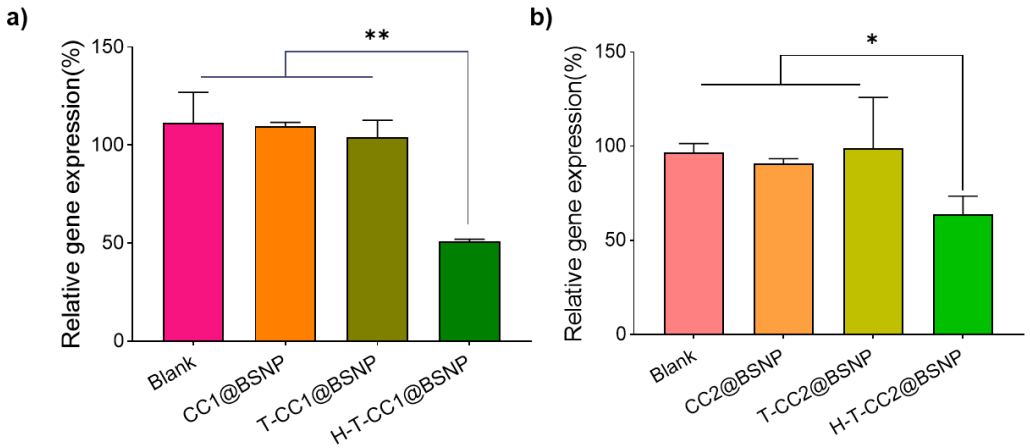

Figure S14. qPCR detection of (a) *COX1* and (b) *COX2* gene expression levels in HeLa cells after treatment with the indicated nanoparticles (200 μg mL^{-1}, 48 h). *p<0.05, ***p<0.001.

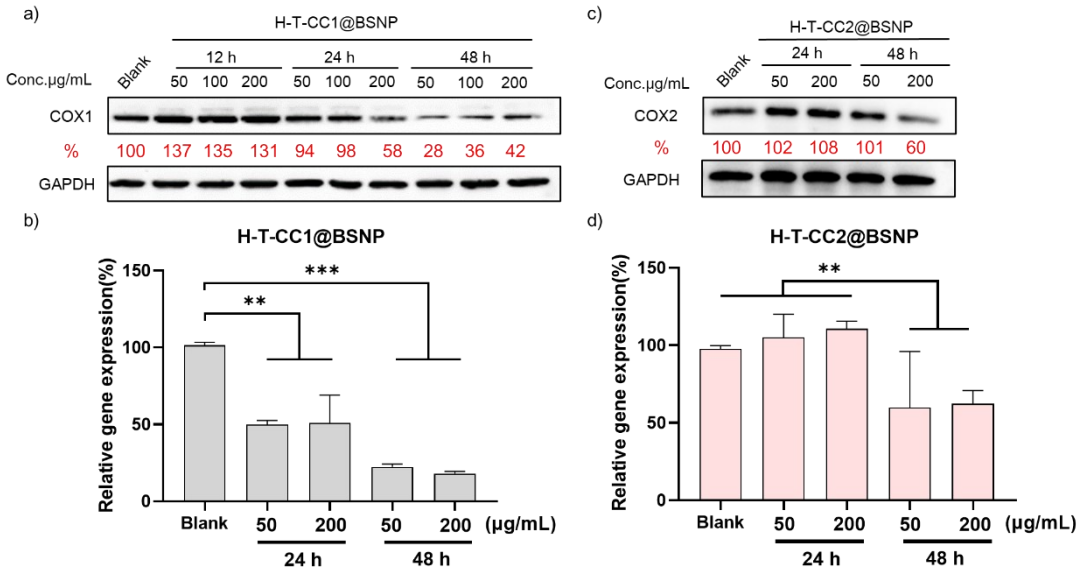

Figure S15. a) HeLa cells were treated with different concentrations of H-T-CC1@BSNP. WB analysis was performed on COX1 proteins and loading controls (GAPDH) after treatment for 12 h, 24 h, 48 h, respectively. b) qPCR analysis of COX1 in HeLa cells after incubation with different concentrations of H-T-CC1@BSNP for different time periods. c) WB and d) qPCR analysis of COX2 in HeLa cells treated with different concentrations of H-T-CC2@BSNP for 24 h, 48 h, respectively. *p<0.05, ***p<0.001.

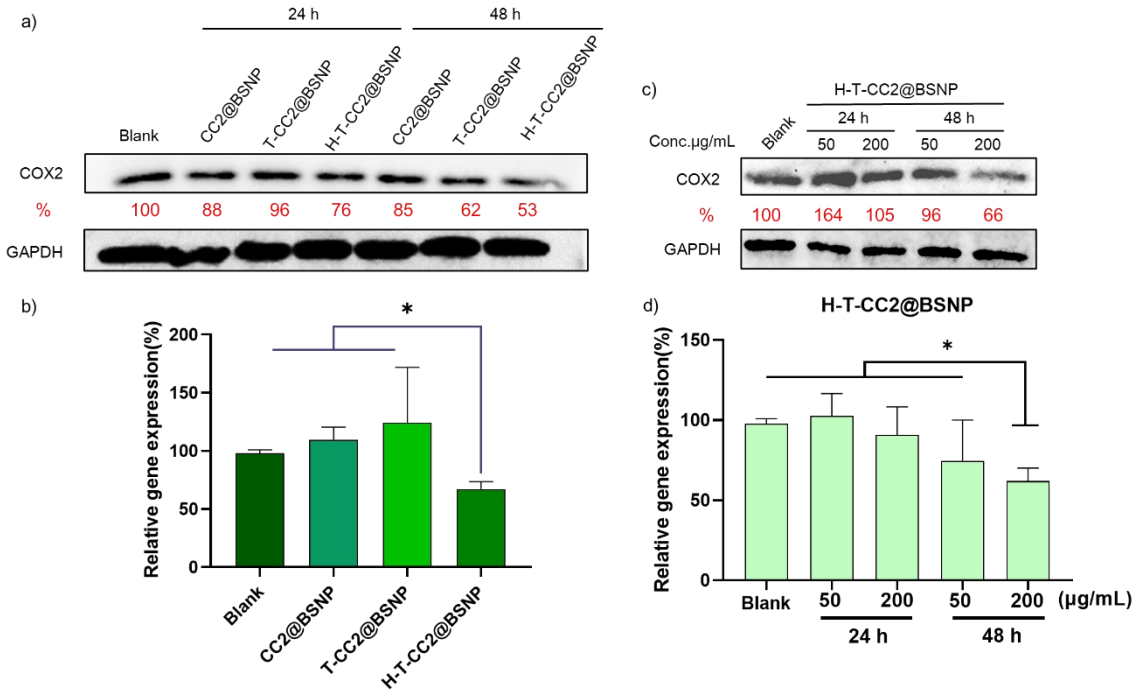

Figure S16. a) WB and b) qPCR analysis of COX2 in A549 cells treated with different CC2-loaded BSNP for 24 h, 48 h, respectively. c) WB and d) qPCR analysis of COX2 in A549 cells treated with different concentrations of H-T-CC2@BSNP for 24 h, 48 h, respectively. *p<0.05, ***p<0.001.

References

1. A. Jo, S. Ham, G. H. Lee, Y. Lee, S. Kim, Y. Lee, J. Shin, Y. Lee and J. Blasiak, *Biomed Res. Int.*, **2015**, 2015, 305716.
2. W. Du, S. Du, X. Dong, H. Bai, J. Jiang, S. Hao, F. Yang, Q. Xiao, B. Zhang, J. Ge, L. Gao, L. Li, S. Q. Yao and W. Huang, *Biomaterials*, **2023**, 294, 122000.
3. G. Chen, A. A. Abdeen, Y. Wang, P. K. Shahi, S. Robertson, R. Xie, M. Suzuki, B. R. Pattnaik, K. Saha and S. Gong, *Nat. Nanotechnol.*, **2019**, 14, 974-980.

Uncropped Western Blot Images