Supplementary Information

DNA lesion-gated dumbbell nanodevices enable on-demand activation of the cGAS-STING pathway for enhancing cancer immunotherapy

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Experimental procedures

Reagents and materials

Apurinic/apyrimidinic endonuclease 1 (APE1), T4 DNA ligase (T4), and uracil glycosylase inhibitor (UGI) were purchased from New England Biolabs Inc. (NEB, USA). 7-nitroindole-2-carboxylicaid (NCA), exonuclease I (Exo I), and exonuclease III (Exo III) were bought from Thermo Fisher Scientific Inc. (Shanghai, China). Uracil DNA glycosylase (UDG), Lipofectamine 3000, and Annexin V-FITC/PI kits were obtained from Vazyme Biotech Co., Ltd. (Nanjing, China). Calcein acetoxymethyl ester (Calcein-AM), cell counting kit-8 (CCK-8), and Hoechst 33342 were obtained from Beyotime Biotech. Inc (Shanghai, China). Propidium iodide (PI) was purchased from Sigma-Aldrich. Enzyme-linked immunosorbent assay (ELISA) kits of human cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) and human IFNβ (RX106216H) were purchased from Dongguan Enzyme-linked Biotechnology Co., Ltd and Ruixin Biotech Co., Ltd. (Quanzhou, China), respectively. Recombinant human IL-2 protein was purchased from Proteintech Group, Inc. Dulbecco's modified eagle medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium were provided by Gibco (Shanghai, China) for cell experiments. Ultrapure water was used with an electric resistance of 18.2 MΩ•cm. The HPLC-purified DNA oligonucleotides were prepared by Hippo Biotech Co., Ltd. (Zhejiang, China), and the sequences are listed in Table S1. Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) and Tris-HCl buffer (20 mM Tris, 140 mM NaCl, 1 mM $MgCl_2$, pH 7.4) were used to store and prepare various DNA samples, respectively. All oligonucleotides were centrifuged at 12000 rpm for 20 min, and then stored in TE buffer.

Apparatus

Polyacrylamide gel electrophoresis (PAGE) experiments were performed and imaged by the BG-verMIDI standard vertical electrophoresis apparatus and

a Gel Doc XR$^+$ System (Bio-Rad, California, USA). Fluorescence measurements were carried out by a FL-7100 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). An ultra-high speed and super-resolution real-time dynamic imaging system (SpinSR, Olympus, Tokyo, Japan) was used to perform fluorescence imaging of cells. ELISA experiments and cytotoxicity study were tested by a SpectraMax Synergy H1 microplate reader (BioTek, USA). Flow cytometry experiments were performed by a FACS Verse11000 (Becton, Dickinson and Company, USA).

Preparation of UBLE

Before use, oligonucleotides were dissolved in Tris-HCl buffer at a final concentration of 4 μM. First, the phosphorylated DNA (p-DNA) was heated at 95°C for 5 min and then cooled to 4°C to obtain a stable middle-opened dumbbell nanodevice (MOD). The MOD was next incubated with 4 U/μL of T4 (with 0.5 x T4 DNA ligase buffer) at 16°C overnight for ligation and then heated at 65°C for 20 min for T4 denaturation. Finally, Exo I (1 U/μL) and Exo III (2 U/μL) with their corresponding buffers (1 x) were added to the above mixture to degrade any unconnected MOD and hybrid dsDNA by-products at 37°C for 2 h. After denaturation of Exo I and Exo III by heating at 80°C for 20 min, the pure UBLE was formed and then stored at 4°C for subsequent use. The nBLE was also prepared following the same procedures of UBLE as controls. Other dumbbell probes (1-OD and 2-OD) and hairpin probes (SP and TP) were heated at 95°C for 5 min and then cooled to 4°C to obtain a stable structure for comparisons.

Gel electrophoresis analysis

To evaluate the formation of UBLE and the long dsDNA self-assembly, PAGE tests were employed to analyze the results. For the feasibility of UBLE formation, the different DNA samples (10 μL) were prepared with a final concentration of 500 nM after being treated with T4 or Exo I/III. To evaluate the dsDNA self-assembly, the prepared UBLE (500 nM, 10 μL) was treated with

UDG and APE1 alone or together for samples. Then, these prepared samples were mixed with 2 μL of loading buffer (6×), and then loaded into an 8% freshly prepared polyacrylamide gel. Subsequently, the electrophoresis was performed in 1× TBE buffer at 120 V for 40 min. Finally, the gel was stained with Gel-Red for 15 min and photographed by Gel Doc XR$^+$ System for image analysis.

Decay kinetics evaluation of UBLE stability

To assess the structural stability of UBLE, PAGE tests were performed to analyze the degradation rates. For each structure, we performed a series of tests at different incubation times. First, UBLE and other comparative probes (1-OD, 2-OD, SP, and TP) were prepared with a concentration of 2.5 μM. Then, 2 μL of these probes were added to 8 μL of Exo I/III mixture (1 U/μL of Exo I and 2 U/μL of Exo III) or 10% fetal bovine serum (FBS) to incubate at 37 °C for different time intervals (final of 500 nM, 10 μL). Finally, these samples were carried out in the PAGE procedures as the above operations.

According to the previously reported work,[1] we quantified the exposure image of different structures by Image J software and employed an exponential decay model to obtain the time constants for each structure in Figs. S2 and S3. Finally, we divided the time constants of UBLE by 1-OD and 2-OD to obtain the corresponding folds for evaluating UBLE stability (Figs. 2d and 2e).

Förster resonance energy transfer (FRET) assays

To assess the feasibility of UBLE in self-assembling long dsDNA, we carried out a series of fluorescence assays. For feasibility response of UBLE to UDG, 40 μL of UBLE (100 nM) and UDG (50 U/mL) were reacted at 37°C for 1 h. The FRET signals for dsDNA were then recorded with an emission range from 550 nm to 800 nm on the excitation of 540 nm, using a PMT voltage set at 900 V and a slit width of 5.0 nm. For the kinetic analysis of UBLE, the fluorescent signals were recorded by real-time monitoring at above same conditions. Additionally, the nBLE was used as a control to operate the same experiments for comparisons.

After the feasibility of long dsDNA self-assembly, we then evaluated the response to different concentrations of UDG and the inhibition effect of UGI. The prepared UBLE (final of 100 nM, 40 μL) was co-incubated with varying concentrations of UDG (0, 0.01, 0.05, 0.1, 0.5, 1, 2, 5, 7.5, 10, 20, 50 U/mL) for FRET assays (where the concentration of APE1 is consistent with that of UDG). For the inhibition effect test, the UDG (10 U/mL) was first incubated with different concentrations of UGI (ranging from 0, 0.5, 1, 2, 5, 10, 20, and 50 U/mL) at 37°C for 1 h, and then reacted with UBLE at 37°C for another 1 h to get signal recordings. Moreover, the specificity of UBLE was also measured by testing the response of other interfering enzymes with a final concentration of 10 U/mL, including alkane-inducible protein B (AlkB), nicking endonuclease (Nt. BbvCI), deoxyribonuclease I from diplococcus pneumonia (DpnI), flap endonuclease 1 (FEN1), human alkyladenine DNA glycosylase (hAAG).

Notably, the FRET signals for dsDNA were obtained by dividing the Cy5 intensity by Cy3 intensity (i.e., F_{cy5} / F_{cy3}) throughout the whole experiment.

Calculation of the detection limit

As the varying concentrations of UDG from 0 to 50 U/mL, the FRET signals for dsDNA were detected with a gradually increased tendency (Fig. S7). Calibration analysis showed a great linear correlation between the FRET signals for dsDNA and the logarithm of UDG in the range of 0.5-10 U/mL (Fig. 3f). The linear regression equation was derived as $y = 0.3011 \lg c + 0.1848$ and the coefficient of determination was $R^2 = 0.9983$. According to the calibration curve, we calculated the limit of detection (LOD) following equation,[2] where I_B is the FRET signals of dsDNA in the blank sample and S_B is the standard deviation of blank samples.

$$I_L = I_B + kS_B$$

In this work, the FRET signals of the blank sample were executed with three parallel experiments, exhibiting an average FRET signal of dsDNA in the blank sample (I_B) was 0.0815 and a standard deviation (S_B) was 0.001. Finally,

under a signal-to-noise ratio value (k) of 3, the smallest detectable signal could be calculated as I_L= 0.0845, and the LOD is calculated to be 0.46 U/mL.

Cell culture

HeLa cells (human cervical cancer cell line and LO2 cells (human normal hepatocytes) were acquired from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Cells were separately cultured in 25 cm^2 T-type (T-25) flasks and maintained at subconfluent density at 37 °C in a humidified atmosphere (5% CO_2). The used medium was DMEM with 10% fetal calf serum (FBS), and 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin). Upon reaching cell confluency over 80%, cell subculturing would be performed to sustain optimal growth for subsequent use. Human natural killer cells (NK-92 cells) were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% antibiotics, and 200 IU/mL IL-2 in a humidified atmosphere at 37 °C with 5% CO2, and fresh medium was replaced every 2 days.

Fluorescence imaging analysis of selection activation on UBLE

HeLa and LO2 cells were respectively introduced into a 35-mm confocal dish and cultured in DMEM at 37 °C within a humidified atmosphere (5% CO_2) to reach 80% confluency. For validation of the selection activation of UBLE, the DNA samples (liposome 3000 mixed with 100 nM of UBLE or nBLE following the manufacturer's instruction) were then incubated with HeLa or LO2 cells for 8 h to achieve sufficient cell uptake. Subsequently, these cells were stained with 30 μL of Hoechst 33342 solution for 20 min and then washed three times with PBS. Finally, the cells were cultured in 1 mL of DMEM medium for fluorescence imaging by using the real-time dynamic imaging system (SpinSR, Olympus, Tokyo, Japan). For the assays of the inhibition effect on UDG and APE1, HeLa and LO2 cells were both first pre-treated with 50 U/mL of UDG inhibitor (UGI) and 100 μM of APE1 inhibitor (NCA) for 2 h, and then incubated with UBLE samples for 8 h to perform imaging analysis as negative controls. For the LO2 positive control sets, different from the HeLa cells directly incubated with UBLE

for imaging, these LO2 cells were first pre-incubated with 50 U/mL of UDG and APE1 for 2 h due to the lack of UDG and APE1 and then incubated with UBLE at another 8 h for the positive control. For quantitative analysis of the FRET signals for dsDNA, these fluorescence intensities were analyzed by Image J software, and the FRET signals were obtained by dividing the Cy5 intensity by Cy3 intensity for comparisons.

CCK-8 assay for cell cytotoxicity analysis

CCK-8 assay (Beyotime Biotech. Inc.) was carried out to assess cell viability after incubation with UBLE or nBLE. Briefly, HeLa and LO2 cells were separately seeded in a 96-well plate with a density of 3000 cells per well and cultured for 24 h in DMEM. Subsequently, UBLE and nBLE at different concentrations (0, 50, 100, 200 nM) were incubated with these cells for another 36 h. Finally, the cells were treated using CCK-8 solution according to the manufacturer's recommendations and then monitored the absorbance at 450 nm by a SpectraMax Synergy H1 microplate reader (BioTek, USA).

Calcein-AM/PI staining analysis

To visually assess the impact of UBLE on cell viability, a Calcein-AM/PI staining assay was employed to distinguish live and dead cells. HeLa cells were seeded in a 35-mm confocal dish and cultured to achieve a confluency of 80% in DMEM. Then, the cells were incubated with different samples (liposome 3000 mixed with 200 nM of UBLE or nBLE) for 36 h. Subsequently, the culture medium was discarded and the cells were washed with PBS three times slightly. Finally, after the incubation with Calcein-AM and PI for 15 min, the cells were washed and analyzed by the real-time dynamic imaging system (SpinSR, Olympus, Tokyo, Japan). Besides, these cells without treatments of UBLE and nBLE were also performed to monitor their viability as controls.

Analysis of cGAMP and IFN-β by ELISA

ELISA was employed to measure the cGAMP abundance occurring in the cGAS-STING pathway and the production of IFN-β for enhancing cancer immunotherapy. For cGAMP abundance detection, after incubation with 400 nM of UBLE or nBLE for 12 h in DMEM, HeLa cells were collected. Following that, the extractions were performed by the Human cGAMP ELISA Kit (Dongguan Enzyme-linked Biotechnology Co., Ltd) according to the manufacturer's instructions and then measured the abundance of cGAMP at 450 nm by a SpectraMax Synergy H1 microplate reader (BioTek, USA). Similarly, for IFN-β measurements, the culture medium of HeLa cells was centrifuged and collected after incubation with 400 nM of UBLE for 36 h. The production levels of IFN-β were detected by the human IFN-β ELISA Kit (Ruixin Biotech Co., Ltd. Cat#RX106216H) according to the manufacturer's protocols. Finally, the absorbance value at 450 nm was also measured by using a SpectraMax Synergy H1 microplate reader (BioTek, USA). As a control, cells without treatments of UBLE and nBLE were also performed to monitor the corresponding levels.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis of IFN-β mRNA

To measure the mRNA levels of IFN-β, TNF-α, and IL-6, when incubated 400 nM of UBLE or nBLE with HeLa cells, the total RNA was extracted using an RNA Extraction Kit (Hangzhou Bioer Technology Co., Ltd.) by following the manufacturer's instructions. Next, the complementary DNA (cDNA) was synthesized via the extracted RNA using PerfectStart Green qPCR SuperMix (TransGen Biotech). Finally, the obtained cDNA was used to perform the qPCR reaction system by using ToloScript All-in-one RT EasyMix (Tolo Biotech Co., Ltd.) after adding the specific primers of IFN-β, TNF-α, and IL-6. The β-actin primer pairs were used as housekeeping genes for normalizing the mRNA values, and the corresponding gene sequences were displayed in Table S1.

Results and discussion

Preparation and characterization of UBLE

Fig. S1 Schematic diagram of DNA bases of UBLE (without open sides) and other two dumbbell probes of 1-OD (with one open side) and 2-OD (with two open sides), respectively.

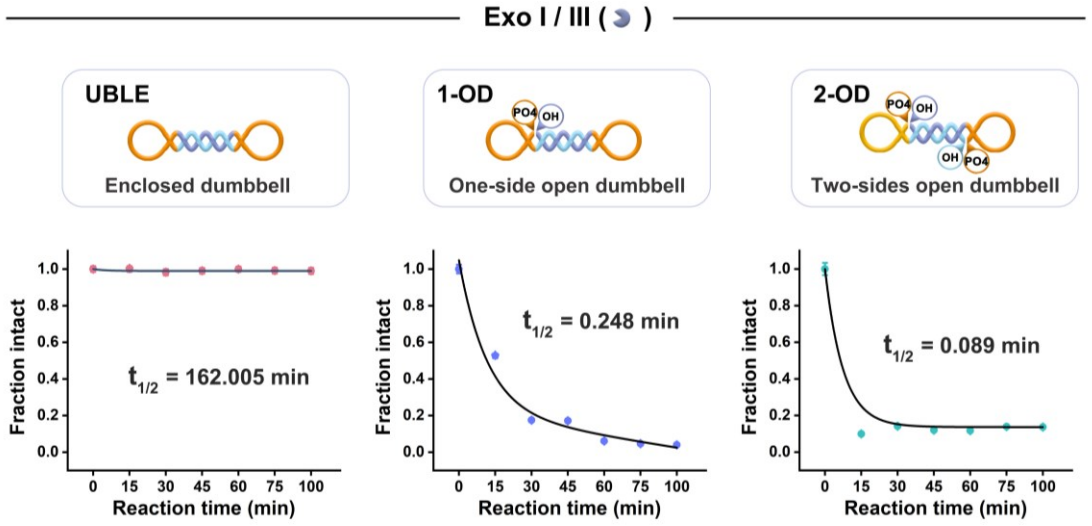

Fig. S2 Normalized decay kinetics of different dumbbell probes in Exo I/III fitted with an exponential decay model for the time constants.

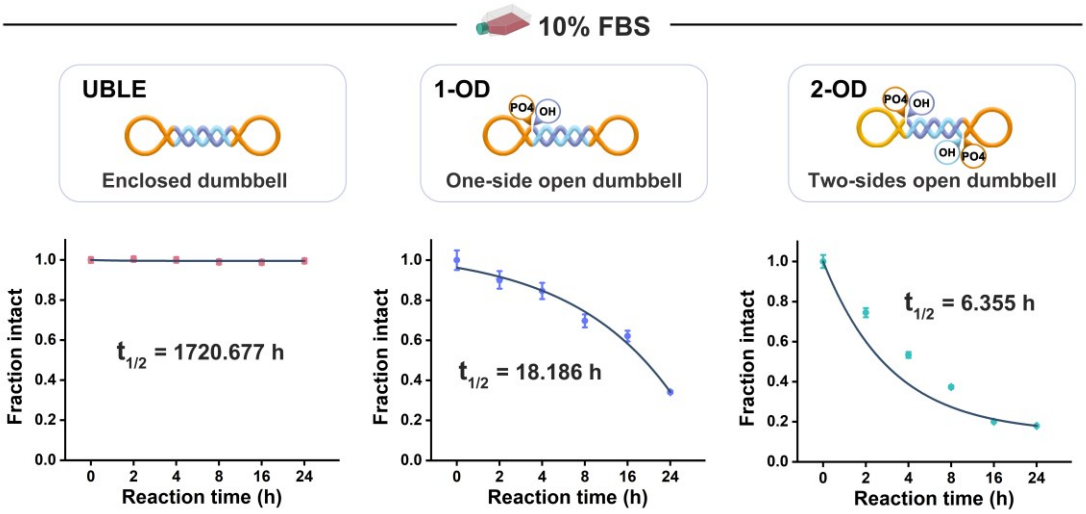

Fig. S3 Normalized decay kinetics of different dumbbell probes in 10% FBS by fitted with an exponential decay model for the time constants.

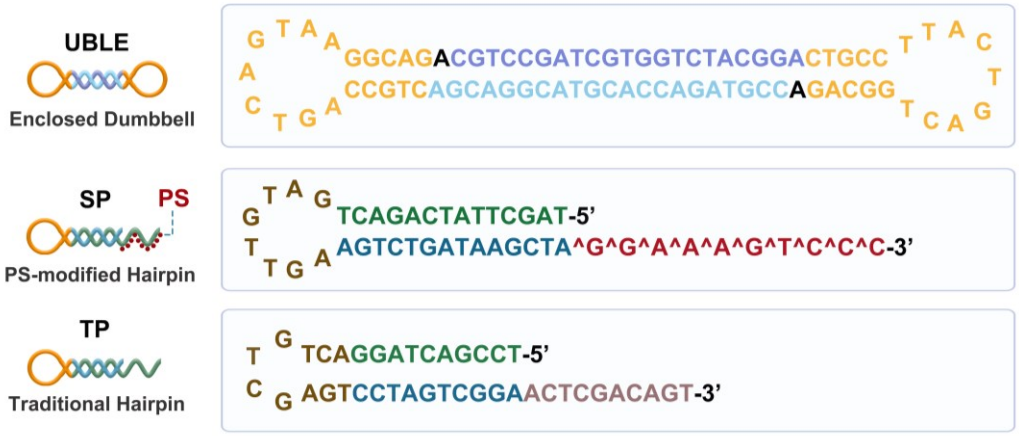

Fig. S4 Schematic diagram of DNA bases of UBLE (without open sides) and other two hairpin probes of SP (phosphorylation-modified hairpin) and TP (traditional hairpin without modifications), respectively.

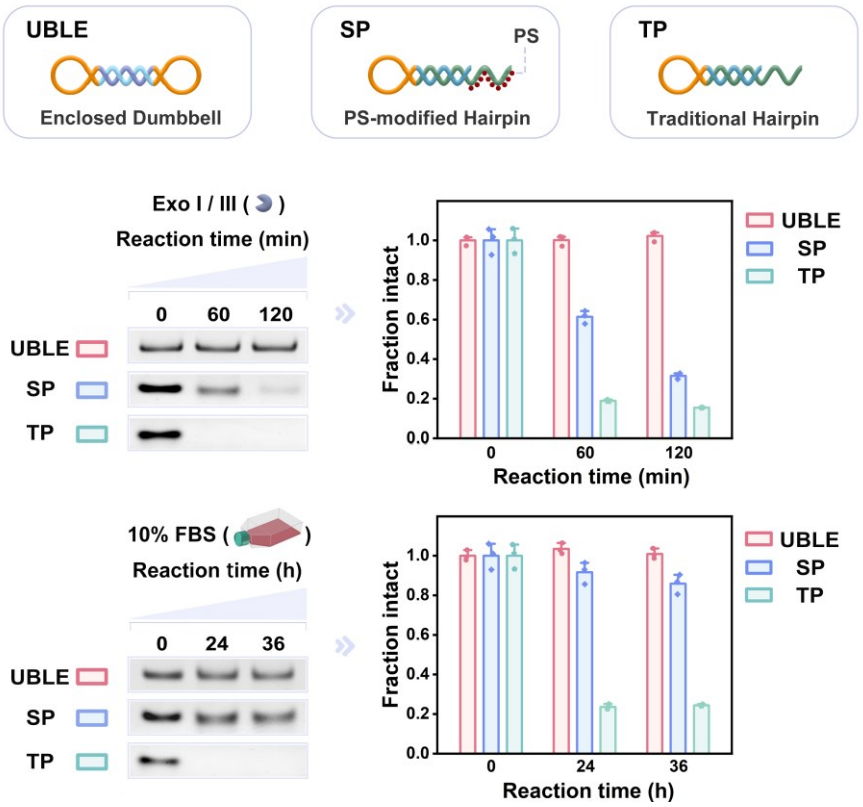

Fig. S5 Resistance comparison of UBLE, SP, and TP by treated with Exo I/III or 10% FBS at different time points.

On-demand activation of UBLE by DNA lesion repair enzymes

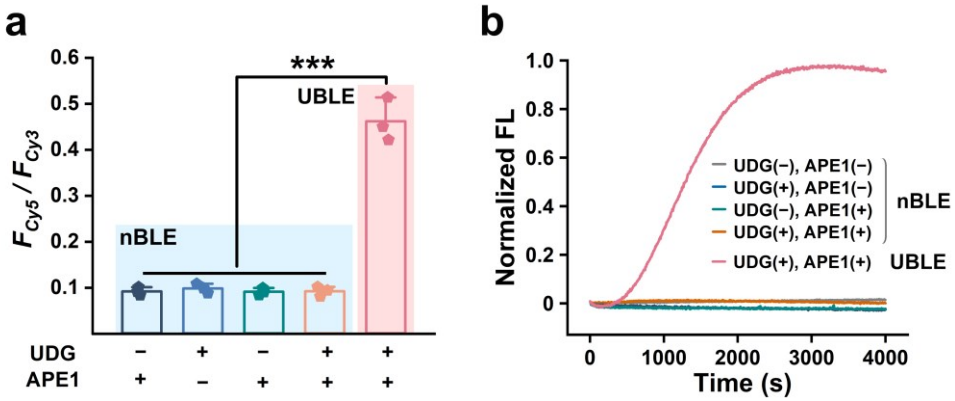

Fig. S6 (a) FRET responses and (b) fluorescence kinetic analysis of nBLE in the presence of UDG and APE1 alone or together compared to the UBLE in the presence of UDG and APE1. Data are represented as mean ± SD (n = 3). *** p < 0.001 by two-tailed Student's t-test. (The positive control of UBLE in the presence of UDG and APE1 are also shown in Figs. 3d and 3e of the main text).

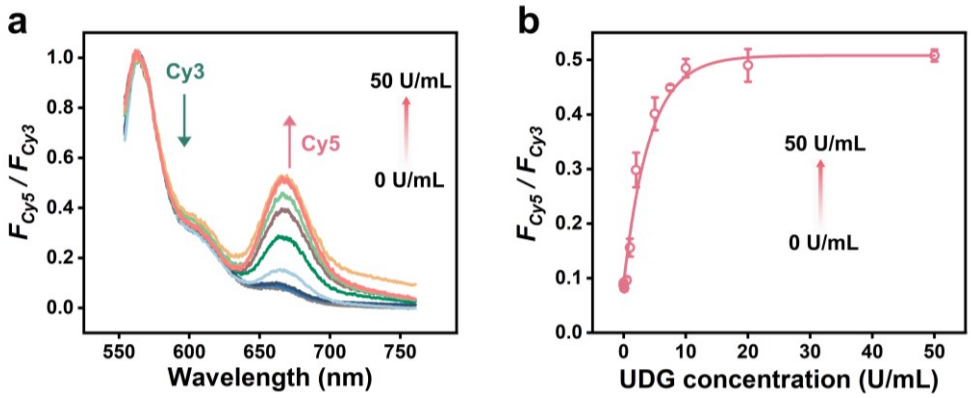

Fig. S7 (a) FRET response analysis of UBLE in response to different concentrations of UDG from 0 to 50 U/mL (where the concentration of APE1 is consistent with UDG). (b) Corresponding calibration curves for quantifying the FRET response in the range of UDG concentrations from 0 to 50 U/mL.

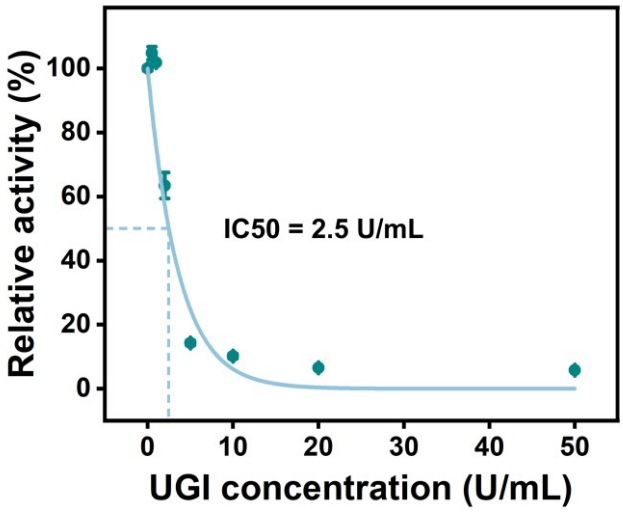

Fig. S8 FRET responses for evaluation of the inhibition effect at various concentrations of UGI (0, 0.5, 1, 2, 5, 10, 20, 50 U/mL), and the half maximal inhibitory concentration (IC50) of UGI is calculated with a value of 2.5 U/mL.

Validation of DNA lesion-gated self-assembly of UBLE

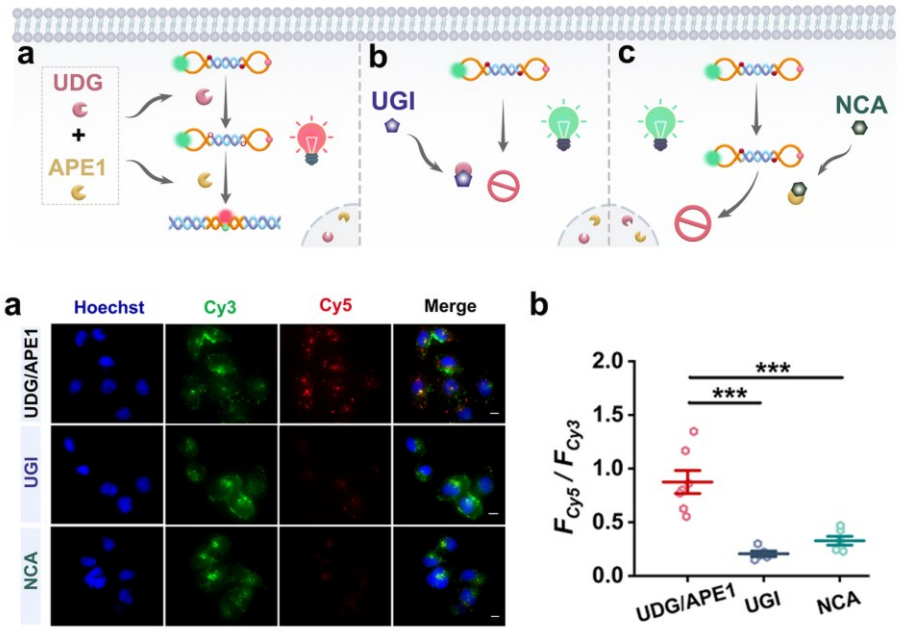

Fig. S9 (a) Schematic illustration of the inhibitor analysis in LO2 cells treated with UDG/APE1, UGI, and NCA, respectively. (b, c) Fluorescence imaging (b) and quantitative analysis (c) of LO2 cells by treatment with UDG/APE1, UGI, and NCA, respectively. Scale bars: 10 μm. Data are represented as mean ± SD. *** $p < 0.001$ by two-tailed Student's *t*-test.

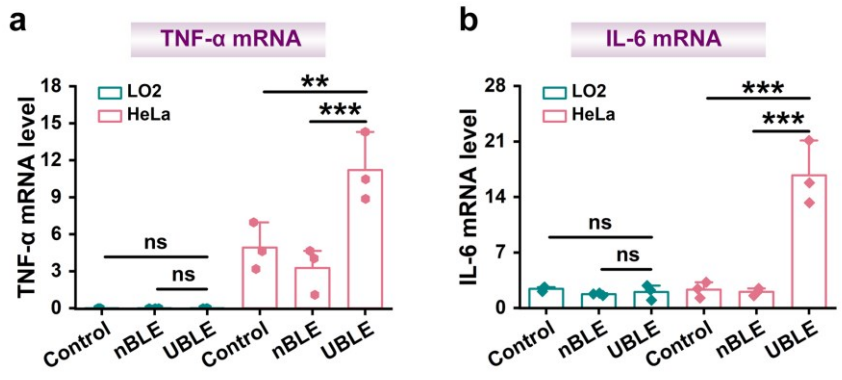

Fig. S10 Relative mRNA expression levels of TNF-α (a) and IL-6 (b) quantified from RT-qPCR. Data are represented as mean ± SD ($n = 3$). *ns*, not significant, ** $p < 0.01$ and *** $p < 0.001$ by two-tailed Student's *t*-test.

Table S1. The sequences of oligonucleotides were used in this work

Name	Sequences(5'-3')
UBLE	pho-GCATGCCTGCUGACGGAATGACTGACCGTCAGCAGGCATGCACCAGATGCCUGACGGTCAGTCATTCCGTCAGGCATCTGGT
UBLE-F	pho-GCATGCCTGCUGACGGAATGA/Cy3/CTGACCGTCAGCAGGCATGCACCAGATGCCUGACGGTCAGT/Cy5/CATTCCGTCAGGCATCTGGT
nBLE	pho-GCATGCCTGCAGACGGAATGACTGACCGTCAGCAGGCATGCACCAGATGCCAGACGGTCAGTCATTCCGTCAGGCATCTGGT
nBLE-F	pho-GCATGCCTGCAGACGGAATGA/Cy3/CTGACCGTCAGCAGGCATGCACCAGATGCCAGACGGTCAGT/Cy5/CATTCCGTCAGGCATCTGGT
1-OD	GACGGAATGACTGACCGTCAGCAGGCATGCACCAGATGCCAGACGGTCAGTCATTCCGTCAGGCATCTGGTGCATGCCTGC
2-OD-l	GACGGAATGACTGACCGTCAGCAGGCATGCACCAGATGCC
2-OD-r	GACGG*AATGACTGA*CCGTCAGGCATCTGGTGCATGCCTGC
SP	TAGCTTATCAGACTGATGTTGAAGTCTGATAAGCTA^G^G^A^A^A^G^T^C^C^C
TP	TCCGACTAGGACTGTCGAGTCCTAGTCGGAACTCGACAGT
IFN-β F	GACCAACAAGTGTCTCCTCCAAA
IFN-β R	AGCAAGTTGTAGCTCATGGAAAGAG
TNF-α F	CCTGGAAAGGACACCATGAGC
TNF-α R	CCCCTCAGCTTGAGGGTTTG
IL-6 F	GGTCCAGTTGCCTTCTCCCTG
IL-6 R	CAGCCATCTTTGGAAGGTTCAGG
β-actin F	TAGTTGCGTTACACCCTTTCTTG
β-actin R	TCACCTTCACCGTTCCAGTTT

Notes: (1) The **UBLE-F** stands for **UBLE** modified with the Cy3 and Cy5 molecular. (2) The dumbbell probe of 2-OD was obtained by hybridizing 2-OD-l and 2-OD-r. (3) **^** represents the phosphorylation (PS) modification in the DNA hairpin.

References

[1] A. R. Chandrasekaran, J. Vilcapoma, P. Dey, S. W. Wong-Deyrup, B. K. Dey and K. Halvorsen, *J. Am. Chem. Soc.*, 2020, **142**, 6814-6821.

[2] A. E. Radi, J. L. A. Sánchez, E. Baldrich and C. K. O'Sullivan, *J. Am. Chem. Soc.*, 2006, **128**, 117-124.