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Supporting Information

A carrier-free nanoparticle with dual NIR/acid responsiveness by co-assembly of enediyne and IR820 for combined PTT/chemo therapy

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Materials

Toluene, tetrahydrofuran (THF), and dichloromethane (DCM) were dried over calcium hydride (CaH_2) and distilled before use. IR820 and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT) were purchased from Macklin (Shanghai, China). Annexin V-FITC/PI apoptosis detection kit was purchased from Solarbio, Beijing, China. Alexa Fluor 555-labeled Donkey Anti-Rabbit IgG(H+L), phospho-histone H2A.X (Ser139) rabbit monoclonal antibody and acetyl-Histone H2B (Lys20) rabbit monoclonal antibody were purchased from Beyotime Biotechnology. Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 Medium, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were provided by BBI Life Sciences Corporation. Other reagents were purchased at commercial grade and used without further purification. Sonogashira reactions were performed with dry Schlenk technique under nitrogen atmosphere.

Characterization

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DRX-500, or DRX-600 instruments and calibrated using residual undeuterated solvent ($CDCl_3$: δ_H = 7.26 ppm, δ_C = 77.2 ppm; DMSO-d_6: δ_H = 2.54 ppm, δ_C = 40.4 ppm) as an internal reference. High-resolution mass spectra (HR-MS) were obtained on a Micromass LCTTM mass spectrometer using the ESI method. NIR laser (808 nm, HPS3200; B&A Technology Co., Ltd, Shanghai, China) was used. Absorption spectra were collected using a UV−vis spectrophotometer (PerkinElmer Lambda 950). Fluorescence spectra were recorded on a PerkinElmer LS-55 (excited at 350 nm). Electron spin resonance (ESR) measurements were performed with an X-band EMX-8/2.7C ESR spectrometer (Bruker). Cytotoxicity assay was measured by a microplate reader (Thermo Scientific). Fluorescence microscopy images were taken using a confocal laser scanning microscope (CLSM, Nikon). Quantitative flow cytometry was recorded by a flow cytometer (Beckman).

Method

Preparation of Nanoparticles

The assembly of enediyne and IR820 was optimized based on precipitation method. Briefly, 100 μl methanol containing enediyne was dropped into aqueous solution of IR820

with vigorous stirring. After 4 hours consistent stirring, the solution was dialyzed for 24 h to remove the unloaded small molecules (Method A). For Method B, an additional one-hour ultrasonication was performed after stirring. For Method C, enediyne and IR820 were mixed together in methanol and then dropped in stirring ultrapure water, followed by stirring for 4 h, ultrasonication for 1 h, and dialysis for 24 h.

Characterization of Nanoparticles

The particle size and zeta potential of nanoparticles were measured with a Dynamic Light Scattering (DLS) Analyzer (Malvern Zetasizer 3,000 HS) equipped with a 125 mW laser light and operated at λ = 633 nm. All samples were measured at a scattering angle of 90º. The morphology of EICN was observed by JEM-1400 TEM with an acceleration voltage of 100 kV and GeminiSEM 500 operating at 3 kV. UV-vis absorption spectra were recorded on a PerkinElmer Lambda 950. Fluorescence spectra were recorded on a PerkinElmer LS-55 (excited at 350 nm). The drug loading content of EICN was calculated based on a UV–vis absorption method and the calibrating curves of enediyne and IR820 are shown in Figure S3.

Stability Assay

The size distributions and TEM images of EICN (120 μg/mL) in water, PBS (pH 7.4), RPMI, DMEM (with 10% fetal bovine serum) were recorded for a week at determined time. The size distributions and images of EICN before lyophilization, after lyophilization and re-suspended were also recorded to demonstrate the stability of EICN for long-term storage. For the above experiments, samples were placed at room temperature under dark.

Degradation Behaviors of EICN

To study the degradation behaviors of EICN, the size distributions, zeta potentials and TEM images of EICN (120 μg/mL) in PBS (pH 5.5) with or without NIR irradiation were recorded. Solutions were placed at room temperature under dark for 5 h before the measurements. For NIR irradiation group, after the incubation in PBS (pH 5.5) for 4 h, the samples were irradiated for 10 min (808 nm, 1 W/cm^2) and incubated for another 50 min. The fluorescence spectra were also measured to reveal the fluorescence recovery of enediyne and IR820.

Photothermal Effect of EICN

To determine the photothermal effect of EICN, EICN with a concentration of 50 or 200 μg/mL for IR820 in ultrapure water was irradiated for 5 min, and the temperatures of the samples were measured by Digital Thermal Imager of FLIR E64501. For comparisons, the solutions of water, IR820 and enediyne were also investigated. The concentration of

IR820 and enediyne were regulated to the same concentration as that in EICN solution. The power-dependent photothermal effect of EICN was investigated by adjusting the laser power to 0.5, 0.75, 1 and 1.5 W/cm^2.

The photothermal conversion efficiency (η) was calculated according to the fowling equations:

$$Q_{Dis} = \frac{c_D m_D (T_{Max(DW)} - T_{surr})}{\tau_{s(DW)}}$$

$$t = -\tau_s ln$$

$$\tau_s = \frac{m_D c_D}{hA}$$

$$\eta = \frac{hA(T_{Max} - T_{Surr}) - Q_{Dis}}{I(1 - 10^{-A808})}$$

where c_D is the heat capacity of deionized water (DW, 4.2J /g), m_D is the mass of DW (1 g), $T_{max(DW)}$ is the maximum temperature of DW (23.7 ºC), T_{surr} is the surrounding temperature (21.1 ºC), $\tau_{s(DW)}$ is the associated time constant of DW (359), I is the incident laser power (1 W/cm^2), A_{808} is the absorbance of the sample at 808 nm (1.54). Other parameters were acquired in the Figure S11 as shown below.

Free Radical-generating Ability of EICN

DPBF was used to investigate the free radical-generating ability of EICN. The EICN solution was mixed with DPBF in PBS (pH 5.5 or pH 7.4), and the concentrations were adjusted to be enediyne 50 μg/mL, IR820 63 μg/mL, DPBF 200 μg/mL. Immediately after the treatment, 200 μL solution of the samples was transferred to a 96-well plate and subjected to a microplate reader to measure the absorption at 426 nm. At the predetermined time intervals, the absorptions of different samples were recorded. For the NIR irradiation group, the samples were irradiated with an 808 nm laser at 1 W/cm^2.

ESR measurements were applied to study the free radical-generating ability of EICN. A solution of PBN (100 mM) in DMSO was prepared in advance, then EICN in PBS (pH 7.4 or 5.5) was added to ensure a final concentration of 120 μg/mL. Immediately thereafter, the prepared solution (~150 μL) was transferred to a capillary tube, sealed, and placed in 37 ºC water bath. The ESR spectra were recorded after incubation for 18 h. For NIR irradiation group, after incubation for 4 h, NIR irradiation was applied with an 808 nm laser at 1 W/cm^2 for 10 min and the sample was further incubated for 14 h before the measurement.

DNA Cleavage Assay

Firstly, pUC19 plasmid DNA (200 μg/mL) in pH 8 TE solution was mixed with PBS (pH 7.4 or 5.5) at a ratio of 1:17. Then 2 μL enediyne (in DMSO), IR820 (in water) or EICN (in water) were added to 18 μL PBS containing DNA. The final concentrations were 70 μg/mL for enediyne, 90 μg/mL for IR820, 10 μg /mL for DNA. Meanwhile 2 μL water was added to 18 μL DNA in PBS as the control. All the samples were incubated at 37 ºC for 24 h. For irradiation group, after 4 h incubation, additional irradiation with an 808 nm laser at 1 W/cm^2 for 5 min was applied and the samples were continuously incubated for another 20 h before the measurements. After incubation, each sample (10 μL) was mixed with loading buffer (2 μL) and subjected to a 1% agarose gel electrophoresis at 90 V (101 mA) for 60 min, stained by DuRed, and then the gel was photographed on the UV transilluminator (FR-200A) and analyzed by scanning densitometry.

Cell Culture

Hela cancer cells were obtained from the Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China) and cultured in DMEM medium supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin, 10000 U/mL) in a humidified incubator at 37 ºC with 5% CO_2. Cells were cultured to ~50-70% confluency and harvested using 0.25% trypsin before further cell relevant experiments.

Cytotoxicity Assay

The cytotoxicity was determined *via* MTT assay. Briefly, cells were seeded in 96-well at a density of 3000 cells per well in 100 μL of the cell culture medium and incubated overnight for adherence. Then the culture medium was removed and enediyne, IR820 or EICN with fresh medium were added to the wells. Cells without sample treatment were used as a blank control. For irradiation group, after 4 h incubation, cells were irradiated using an NIR laser (1 W/cm^2) for 5 min. After incubation reached to 48 h, 10 μL of sterile filtered MTT stock solution (5 mg/mL) in PBS (pH 7.4) was added to each well and the cells were further incubated for 4 h at 37 ºC to allow the yellow dye to be transformed into blue crystals. Then, the medium was replaced with 150 μL of DMSO to dissolve the dark blue crystals. Finally, the optical density (OD) of each well was measured at 570 nm by microplate reader. The measurement was S3 normalized using culture medium without cells. Cell viability (%) relative to control containing cell culture medium was calculated by [OD]samples/[OD]control.

Cellular Uptake

The internalization of EICN in Hela cells were examined with confocal laser scanning microscopy (CLSM). Hela cells were seeded in glass bottom confocal dishes at a density of 2 × 10^5 per well in 2 mL of DMEM and incubated overnight. After removal of culture

medium, cells were incubated with IR820, enediyne or EICN in 2 mL of DMEM respectively. The final concentrations were 1.25 μg/mL for enediyne, 1.6 μg/mL for IR820. Hela cells without any drug incubation were performed as a blank control. After 8 h of incubation at 37 ºC, the cells were washed three times with PBS. Subsequently, the cells were fixed with 2.5% glutaraldehyde at room temperature for 10 min, and permeabilized with 0.5% Triton X-100 for another 10 min. After that, the cells were washed with PBS, then 400 μL of DAPI solution (15 μg/mL) was added and the cells were cultured at 37 °C for 10 min, followed by washing with PBS for three times, and finally visualized by CLSM (excitation at 408 nm for DAPI, 488 nm for enediyne, 647 nm for IR820). To study the effect of irradiation on the internalization of EICN, after 4 h incubation, cells were irradiated using an NIR laser (1 W/cm^2) for 5 min and the following procedure was conducted as described above.

Cell Apoptosis Assay

Hela cells were seeded in 6-well plates at a density of 2 × 10^5 per well in 2 mL of DMEM and incubated overnight. After washing with PBS, the cells were incubated with IR820, enediyne or EICN at 37 ºC for 24 h. The final concentrations were 2.5 μg/mL for enediyne, 3.1 μg/mL for IR820. Cells without sample treatment were used as a blank control. For irradiation group, after 4 h incubation, cells were irradiated using an NIR laser (1 W/cm^2) for 5 min. At the end of incubation, the cells were collected by trypsinization, washed twice with cold PBS. Then the cells were re-suspended in 400 μL Annexin binding buffer and stained with Annexin V-FITC (5 μL) and PI (5 μL) at room temperature for 15 min before analyzing by flow cytometry.

Intracellular Free Radical, DNA Damage and HDACi Activity Detection

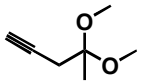
Free radical detection: ROS Assay Kit (Beyotime) with fluorescent probe 2,7-dichloro fluorescein diacetate (DCFH-DA) was used. Cells were cultured using the same procedure mentioned in cell internalization part. After cultured for 8 h, DCFH-DA were added (10 μM) and the dishes were placed in dark for 30 min at room temperature. Then, samples were washed for three times by PBS and subjected to CLSM (excitation at 408 nm for DAPI, 488 nm for DCFH).

DNA damage and HDACi activity: Cells were cultured using the same procedure mentioned in cell internalization part. After cultured for 8 h, cells were fixed, permeated, and further blocked by 8% BSA/PBS for 2 h at room temperature. Then, Cells were stained with the primary antibody (1:200 dilution in 1% BSA/PBS) at 4 ºC overnight. Phospho-histone H2A.X (Ser139) rabbit monoclonal antibody was used as primary antibody for DNA damage detection and acetyl-Histone H2B (Lys20) rabbit monoclonal antibody was used as primary antibody for HDACi activity detection. After the incubation with primary antibody, cells were labeled with secondary antibodies (Alexa Fluor 555-labeled Donkey Anti-Rabbit IgG(H+L), 1:1000 dilution, rt, 2 h). To visualize DNA, cells were further stained with DAPI. Cells were washed three times with PBS after all

incubations (5 min each), after which samples were subjected to CLSM (excitation at 408 nm for DAPI, 561 nm for Alexa Fluor 555).

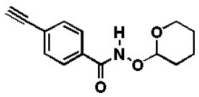
Synthesis

4,4-Dimethoxy-pent-1-yne (Compound 1)



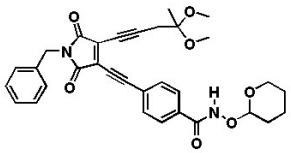
The synthesis of this compound was illustrated in our previous report.[1]
Yellow oil, 2.86 g (55%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.16 (s, 6H), 2.47 (d, J = 2.7 Hz, 2H), 1.98 (t, J = 2.7 Hz, 1H), 1.38 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 99.4, 78.8, 69.3, 47.5, 26.5, 20.5.

4-Ethynyl-N-(tetrahydro-pyran-2-yloxy)-benzamide (Compound 2)[2]



To a solution of 4-ethynyl-benzoic acid (300 mg, 2 mmol), EDCI (452 mg, 2.36 mmol), and HOBt·H_2O (320 mg, 2.36 mmol) in DMF (3 mL) was added NH_2OTHP (346 mg, 2.96 mmol). After the mixture was stirred at room temperature for 24 h, it was poured into water and extracted with ethyl acetate. The organic layer was separated, washed with saturated $NaHCO_3$ and brine, and dried over Na_2SO_4. The product was obtained after purification by silica gel flash column chromatography (ethyl acetate /n-hexane = 1/30) as a yellow solid (448 mg, 90%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.9 (broad, 1H), 7.62 (d, J = 4.0 Hz, 1H), 7.39 (d, J = 4.0 Hz, 1H), 4.96 (s, 1H), 4.77 (s, 1H), 4.05–4.01 (m, 1H), 3.55–3.53 (m, 1H), 1.73–1.71 (m, 3H), 1.55–1.53 (m, 3H).

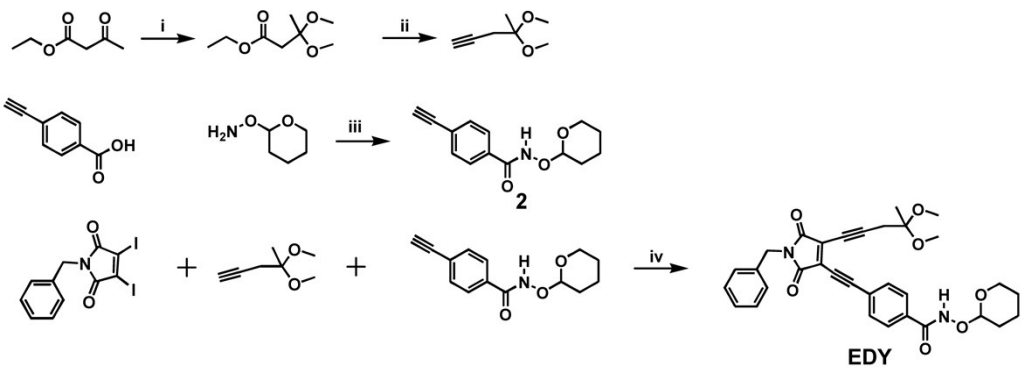
4-[1-Benzyl-4-(4,4-dimethoxy-pent-1-ynyl)-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-ylethynyl]-N-(tetrahydro-pyran-2-yloxy)-benzamide (enediyne)



3,4-Diiodo-N-benzylmaleimide (263.4 mg, 0.6 mmol)[1], CuI (45.7 mg, 40 %), $Pd(PPh_3)_2Cl_2$ (51.0 mg, 12.5 %), K_2CO_3 (248.7 mg, 1.8 mmol) were successively added to a solvent mixture of dry THF (3 mL) and toluene (6 mL) under nitrogen. Then, 1.5 equivalent of compound **1** and **2** in THF (1 mL) were added dropwisely. The mixture was stirred at 40-50 ºC overnight. After the completion of the reaction as detected by TLC, the mixture was directly purified by column chromatography over alkaline silicate (eluted with 5-40% ethyl acetate/hexane) to yield the product as a brown solid (16%).^{1}H NMR (600 MHz, , $CDCl_3$) δ 9.12 (s, 1H), 7.71 (d, *J* = 8.3 Hz, 2H), 7.55 (d, *J* = 8.3 Hz, 2H), 7.31 – 7.19 (m, 5H), 5.00 (s, 1H), 4.64 (s, 2H), 3.97 – 3.90 (m, 1H), 3.62 – 3.53 (m, 1H), 3.17 (s, 6H), 2.86 (s, 2H), 1.85 – 1.74 (m, *J* = 16.1, 7.9 Hz, 3H), 1.61 – 1.50 (m, 3H), 1.44 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.2, 166.7, 166.6,

135.6, 133.1, 132.5, 129.3, 128.7, 128.6, 128.0, 127.7, 127.4, 125.0, 109.0, 107.1, 102.8, 100.5, 81.8, 73.5, 62.7, 48.7, 42.4, 29.6, 28.0, 24.9, 21.8, 18.6. HR-MS (EI): m/z calcd. for $C_{32}H_{32}N_2O_7Na$ (M + Na):579.2107; found: 579.2106.

Supplementary Figures



Scheme S1 Synthesis of compound 1, 2 and enediyne. (i) DIBAL-H, dichloromethane, -78 ºC, 1 h. (ii) Bestmann–Ohira reagent, K_2CO_3, methanol, rt, 24 h. (iii) EDCI, HOBT, DMF, rt, 24 h.(iv) CuI, $Pd(PPh_3)_2Cl_2$, K_2CO_3, tetrahydrofuran, 40-50 ºC， overnight.

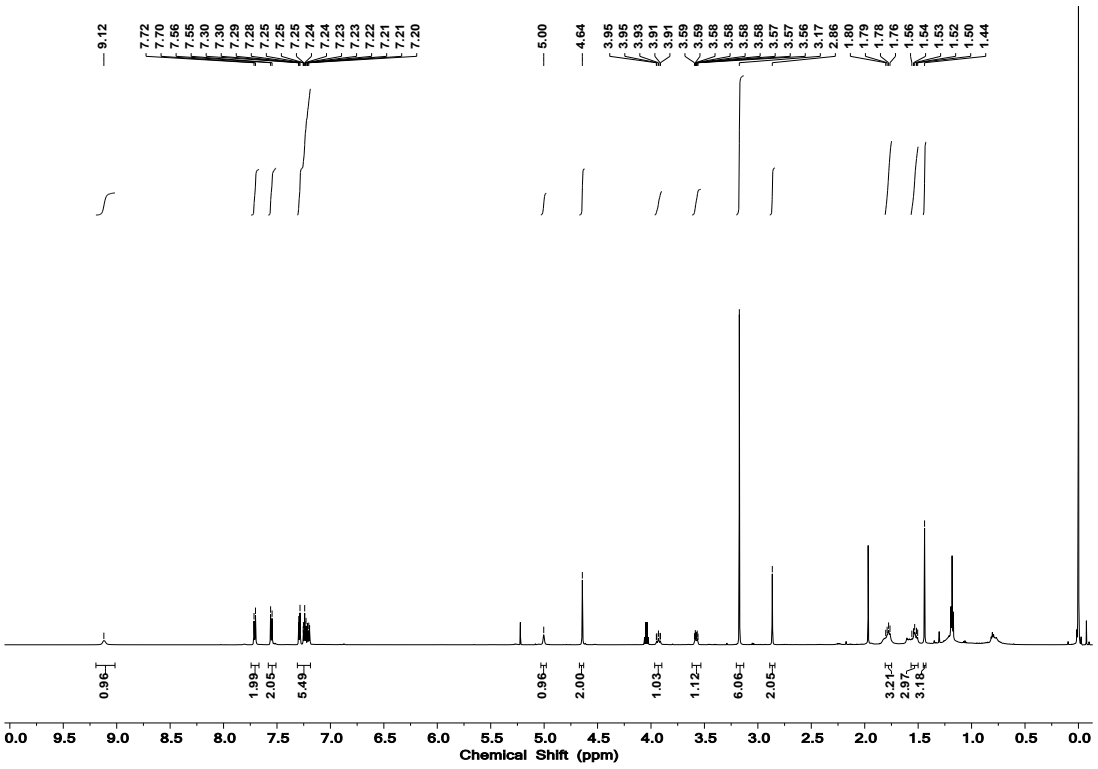


Figure S1. the ^1H NMR spectrum of enediyne.

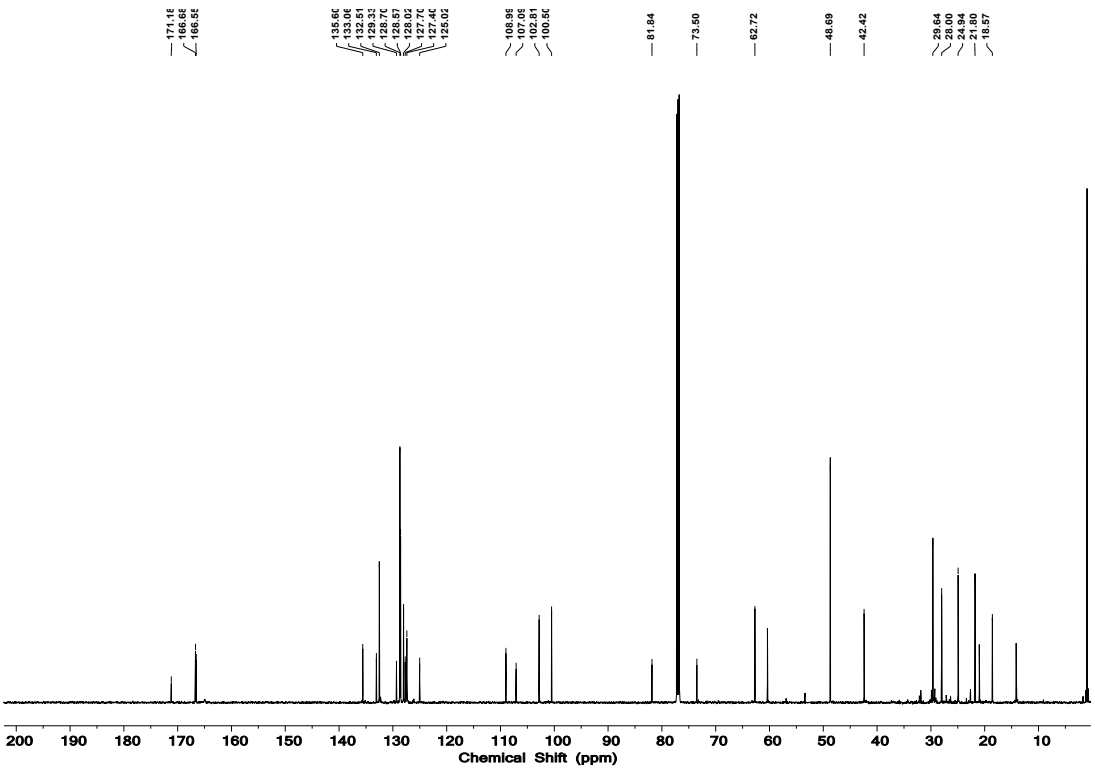


Figure S2. the ^{13}C NMR spectrum of enediyne.

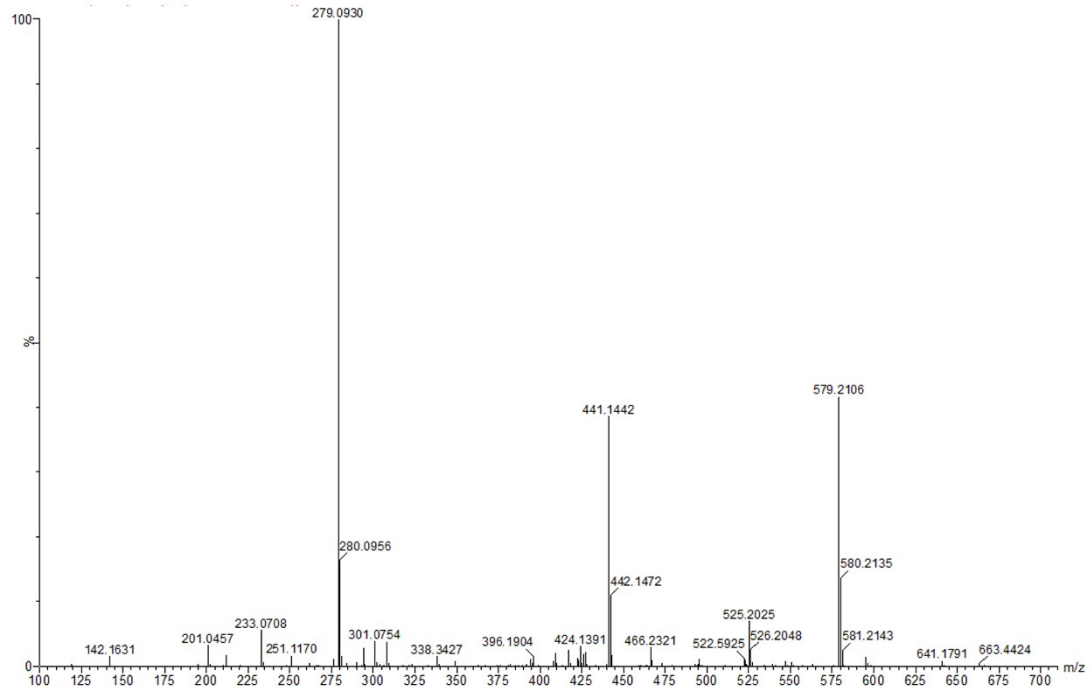


Figure S3. HRMS spectrum of enediyne (calcd. for $C_{32}H_{32}N_2O_7Na$ (M + Na):579.2107; found: 579.2106).

Table S1 Characterization of the nanoparticles prepared by different processes and initial feeding mass ratio of enediyne and IR820.

Input	enediyne:IR820	Size (nm)	PDI	Zeta Potential (mV)	enediyne (μg/mL)	IR820 (μg/mL)	DL (enediyne)	EE (enediyne)	EE (IR820)	Method
1[a]	0.5	214.7	0.846	-15.7	310	617	33%	84%	83%	A
2[b]	1	173.2	0.205	-33.4	421	739	36%	42%	74%	
3	3	159.1	0.098	-30.3	104	196	35%	14%	79%	
4	5	171.2	0.087	-23.5	60	116	34%	8%	78%	
5	1	125.0	0.164	-20.2	530	863	38%	61%	99%	B
6	2	167.0	0.139	-21.9	363	498	50%	49%	98%	
7	3	170.7	0.128	-32.8	278	231	55%	38%	94%	
8	4	182.3	0.151	-28.9	206	132	61%	28%	91%	
9[c]	1	90.4	0.140	-24	568	708	45%	77%	96%	C
10[a,c]	1	110.1	0.227	-23.3	219	382	36%	57%	99%	

[a]The final feed concentration of enediyne in water was 500 mg/mL; [b]the final feed concentration of enediyne in water was 1000 mg/mL for input 2-9. [c]enediyne and IR820 were mixed in methanol.

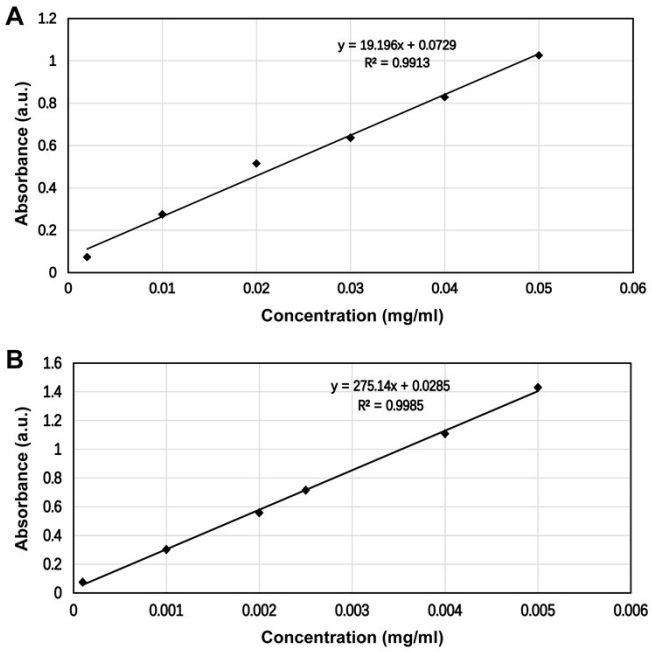


Figure S4. The absorbance of enediyne at 390 nm (A) and IR820 at 790 nm (B) as the function of concentration.

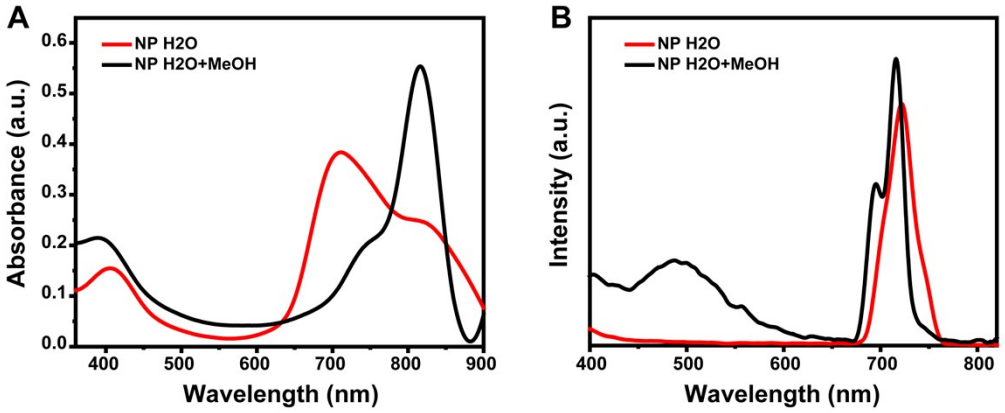


Figure S5. UV-visible absorption spectra (A) and fluorescence spectra (B) of EICN with or without the addition of methanol.

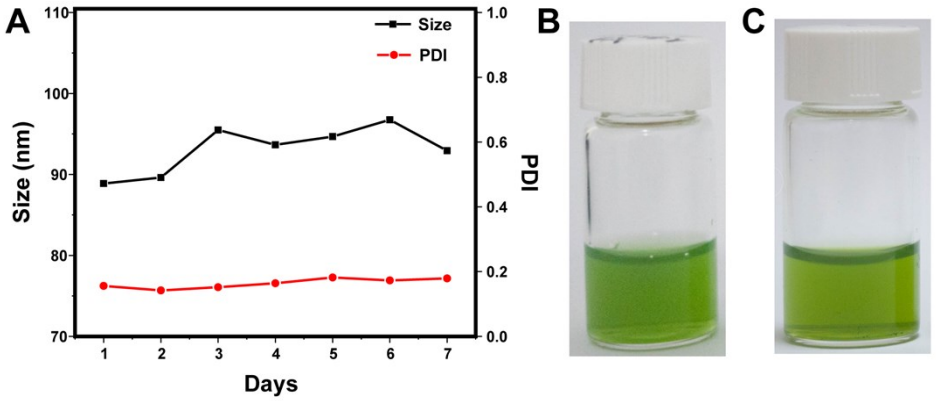


Figure S6. The stability of EICN in UW over time (A); the Images of EICN in water at day one (B) and day seven (C).

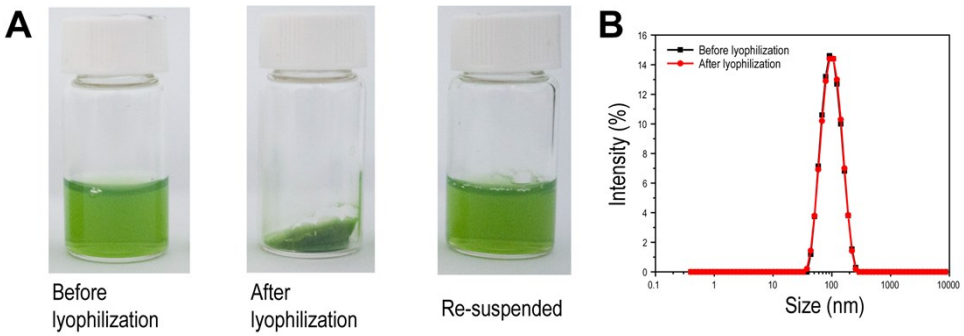


Figure S7. The images of EICN before lyophilization, after lyophilization and re-suspended in water (A). The size distribution of EICN before lyophilization and after re-suspended in water.

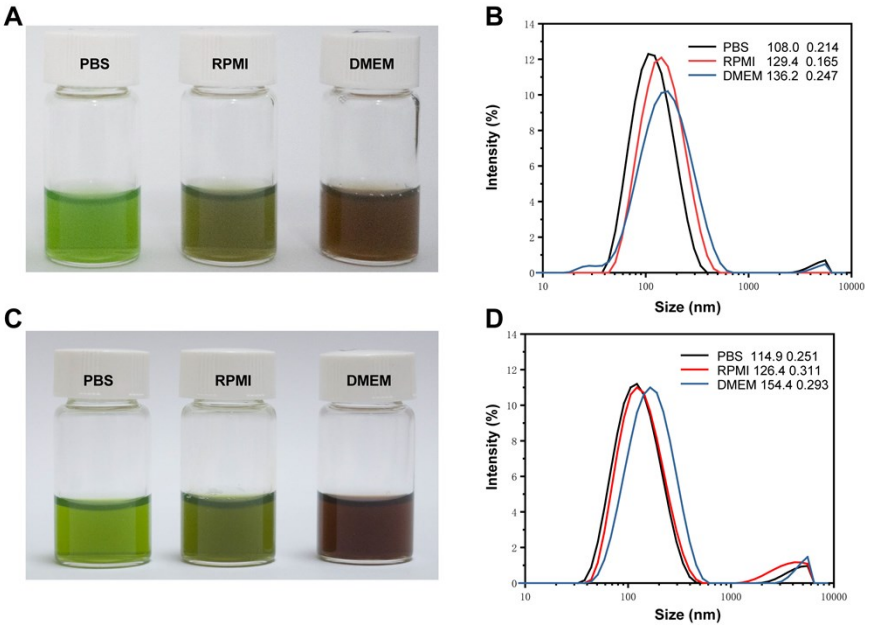


Figure S8. The Images of EICN in PBS, RPMI and DMEM with 10% fetal bovine serum at day one (A) and day seven (C). The size distribution of EICN in PBS, RPMI and DMEM with FBS at day one (B) and day seven (D)

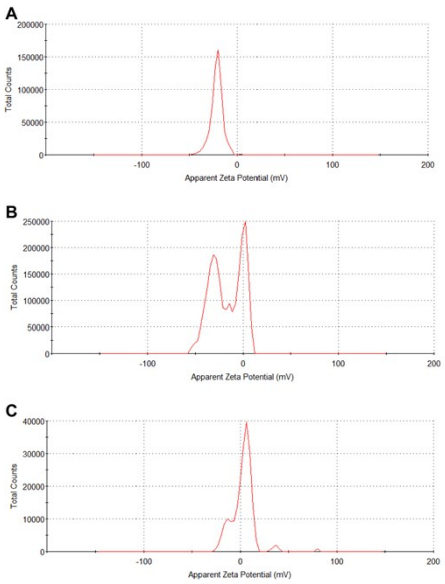


Figure S9. The zeta potential distributions of EICN in water (A), pH 5.5 PBS without (B) or with NIR irradiation (C).

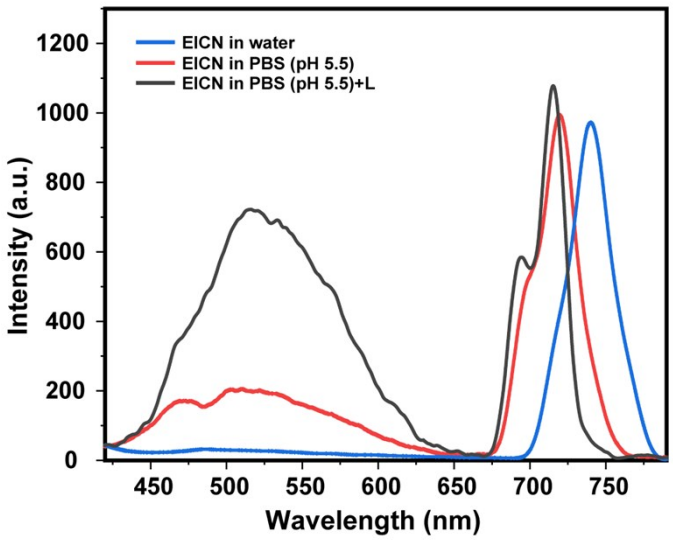


Figure S10. Fluorescence spectra of EICN in water and pH 5.5 PBS with or without irradiation.

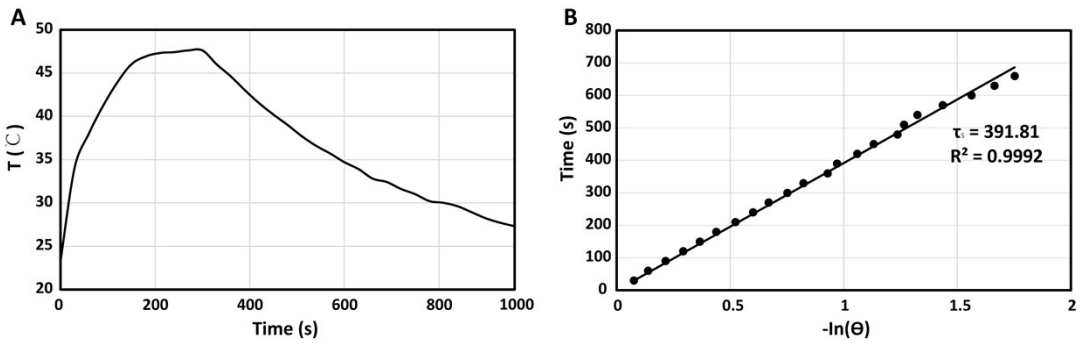


Figure S11. Temperature increase profile of EICN nanoparticles in DW after 300s irradiation with a laser (λ = 808 nm) at power of 1.0 W/cm2 and temperature decrease profile. (d) Linear time data versus -ln(θ) obtained from the cooling period of EICN nanoparticles.

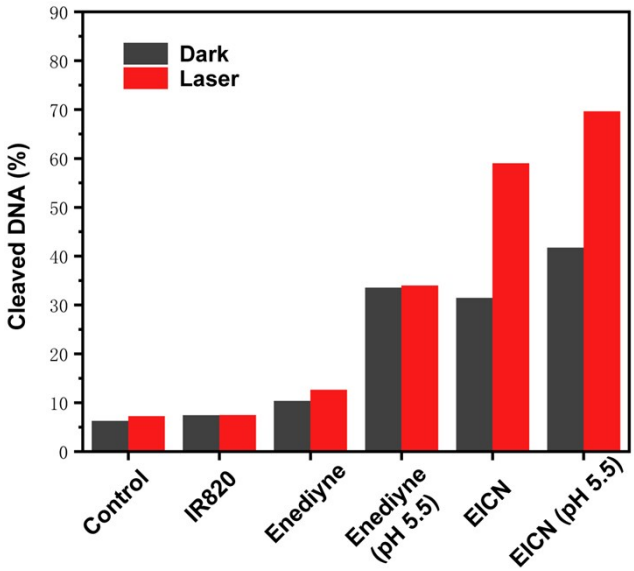


Figure S12. Quantified DNA cleavage percentage for Figure 2 F.

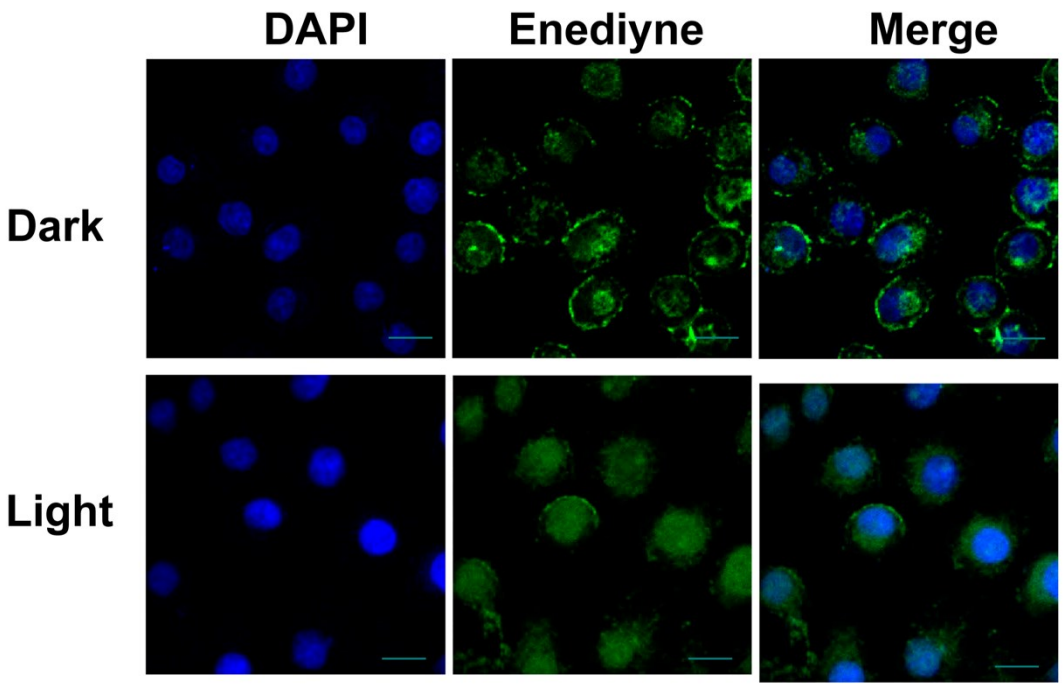


Figure S13. CLSM images of Hela cells incubated with EICN without (up) or with NIR irradiation (down). scale bar: 20 μm.

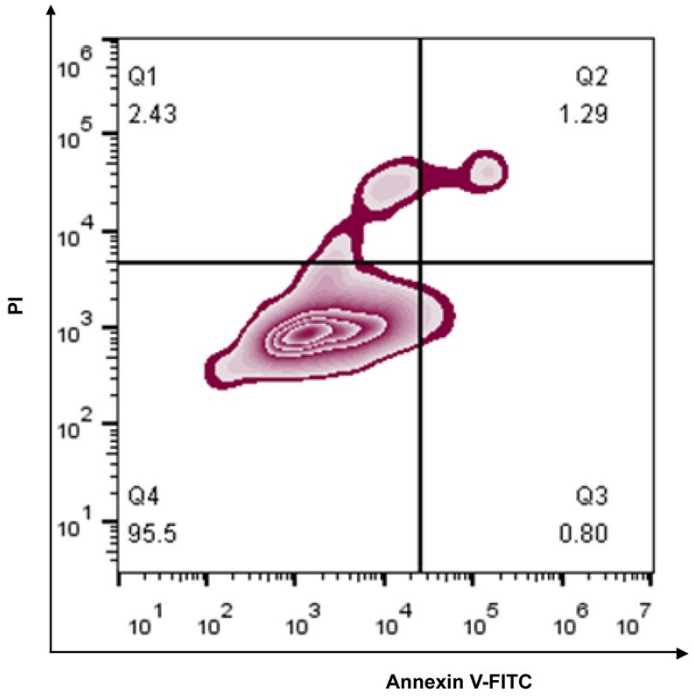


Figure S14. The control group used for apoptosis analysis.

Reference

1. H. Lu, H. Ma, B. Li, M. Zhang, H. Chen, Y. Wang, X. Li, Y. Ding and A. Hu, *J. Mater. Chem. B*, 2020, **8**, 1971-1979.
2. T. Suzuki, Y. Ota, M. Ri, M. Bando, A. Gotoh, Y. Itoh, H. Tsumoto, P. R. Tatum, T. Mizukami, H. Nakagawa, S. Iida, R. Ueda, K. Shirahige and N. Miyata, *J. Med. Chem.*, 2012, **55**, 9562-9575.