Supplementary information

Enhanced Combination Therapy Through Tumor Microenvironment-Activated Cellular Uptake and ROS-Sensitive Drug Release Using a Dual-Sensitive Nanogel

Jianming Yuan, Qinfeng Chen, Mingxiang Zuo, Xiaoxia Li, ChiYi Ou, Qinghua Chen, Dongsheng Yu, Haowen Li, Chenhui Hao, Jing Yang, Shuang Liu, Du Cheng*

Key Laboratory for Polymeric Composite & Functional Materials of Ministry of Education, School of Materials Science and Engineering, Sun Yat-sen University, Guangzhou 510275, PR China

Materials

Acryloyl chloride (99%, J&K) and oxalyl chloride (99%, J&K) were distilled before used. N, N-dimethylaminopropyl acrylamide(98%, Sigma), methyl bromoacetate(98%, Aldrich), cysteamine (99%, J&K), sodium bis(2-ethylhexyl) sulfosuccinate (AOT, 98%, Aldrich), polyethylene glycol lauryl ether (Brij 30, 98%, Aldrich), 2-propionyl-3-methyl-butenedioic anhydride (CDM, 98%, Aldrich), tetramethylethylenediamine (TEMED, 99%, J&K), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 98%, Aldrich), N-hydroxysuccinimide (NHS, 98%, Aldrich), doxorubicin hydrochloride (DOX▪HCl, 99%, MeilunBio) were used as received. Cell penetrating peptide (amino acid sequence, YGRKKRRQRRR; molecular weight, 1559 g/mol; peptide purity, 97%) were synthesized by shanghai Dechi Bioscience Co., Ltd. Dulbecco's modified essential medium (DMEM) and MTT solution were purchased from Thermo Fisher Scientific. Phosphate buffer saline (PBS) solution (0.01 M) was purchased from Beijing Leagene Biotech. Co., Ltd.

Synthesis of carboxybetaine monomer CBAA

N, N-dimethylaminopropyl acrylamide (7.80 g, 50 mmol) was dissolved 30 mL dichloromethane. Methyl bromoacetate (11.48 g, 75 mmol) was the added, also dissolved in 30 mL dichloromethane, and this mixture was added dropwise to the above suspension over a 2-hour period. The reaction was stirred at room temperature for 6 h under a nitrogen atmosphere. After that, the reaction mixture was filtered, and the solid

was washed with anhydrous acetone. The residual solvent was removed by vacuum drying, yielding the pure product (10.85 g) with a yield of 94.8 %.

Synthesis of thioketal crosslinker TK-CL

The synthesis of the TK crosslinker was carried out in two steps. In the first step, mercaptoethylamine (5.68 g, 50 mmol) and anhydrous acetone (7.80 g, 135 mmol) were added to a 100 mL flask, and saturated hydrogen chloride gas was introduced. The mixture was stirred at room temperature for 8 hours. A white precipitate formed, and the solid was filtered and washed twice with chloroform. The crude product was then recrystallized and dried three times to obtain TK-amine (4.21 g) as a pure white solid with a yield of 86%. In the second step, TK-amine (1.94 g, 10 mmol) was dissolved in 20 mL of dichloromethane. Acryloyl chloride (1.98 g, 22 mmol) dissolved in 15 mL dichloromethane was added dropwise to the TK-amine solution under an ice bath. The mixture was stirred for 30 minutes at 0 °C, followed by 4 hours of stirring at room temperature. After monitoring the reaction by TLC, 20 mL of saturated sodium bicarbonate solution was added. The organic phase was extracted, dried with anhydrous magnesium sulfate, concentrated, and purified by column chromatography (ethyl acetate/n-hexane: 1/1) to yield the pure product (1.36 g) with a yield of 45.6%.

Synthesis of polymer PEG-CDM

cis-Aconitic anhydride (CDM) was activated to the acid chloride form using oxalyl chloride. The process is as follows: CDM (3.68 g, 20 mmol) was dissolved in 50 mL of dichloromethane (CH_2Cl_2) and 500 μL of N, N-dimethylformamide (DMF) under a nitrogen atmosphere. While maintaining the solution in an ice bath, 12.70 mL of oxalyl chloride (160 mmol) was added dropwise. The reaction was allowed to proceed for 6 hours at room temperature. Afterward, the organic solution and excess oxalyl chloride were removed by vacuum rotary evaporation, yielding a pale-yellow viscous liquid.

In the next step, $mPEG_{2k}$-OH (0.8 g, 0.4 mmol) was dissolved in 10 mL of CH_2Cl_2 and added to the yellow viscous liquid from the first step. To this mixture, 4-dimethylaminopyridine (DMAP, 96 mg, 0.8 mmol) and triethylamine (TEA, 2.8 mL, 20 mmol) were added, followed by 18 mL of freshly distilled toluene, all while maintaining the solution in an ice bath. The upper layer gradually turned brownish-gray. After the ice bath was removed, the reaction was continued for 36 hours at room temperature. The solution was then filtered under reduced pressure, and the filtrate was concentrated. The product was precipitated in cold ether, reconstituted with 100 mL of CH_2Cl_2, and treated with 30 mL of 0.5 M hydrochloric acid and 60 mL of saturated

sodium chloride solution. The organic phase was collected, dried with anhydrous magnesium sulfate, concentrated, and precipitated again in cold ether to obtain the pale-yellow solid product, $mPEG_{2k}$-CDM.

Synthesis of polymer PEG-CDM-CPP

PEG-CDM-CPP was synthesized through an amidation reaction between cis-aconitic anhydride and a cell-penetrating peptide. Briefly, $mPEG_{2k}$-CDM (0.21 g, 0.1 mmol) was dissolved in anhydrous DMF, and the cell-penetrating peptide (100 mg, 0.064 mmol) was added to this solution. Next, 4-dimethylaminopyridine (DMAP) was dissolved in anhydrous DMSO and added dropwise to the reaction mixture. The solution was stirred for 8 hours. The reaction mixture was then precipitated in cold ether to yield the final solid product (0.29 g) with a yield of 93.5%.

Synthesis of ICG-conjugated BSA (B-ICG)

To prepare ICG-conjugated BSA (B-ICG), 1 g of BSA protein and 400 mg of ICG-NHS were dissolved in purified water. The mixture was stirred for 24 hours at 4 °C, followed by dialysis against pH 7.4 PBS for 72 hours. The resulting dialysate was then lyophilized to obtain 1.2 g of dark blue B-ICG powder.

Optimization of nanogels loaded with ICG-BSA and DOX

The nanogel was prepared according to the procedures outlined in **Tables S1-S4.** Briefly, sodium bis(2-ethylhexyl) sulfosuccinate (AOT) and polyethylene glycol lauryl ether (Brij 30) were mixed with n-hexane in a 50 mL serum bottle. The mixture was vigorously stirred until all reagents were completely dissolved, followed by purging with nitrogen gas for 10 min. For the aqueous phase, 5 mg of B-ICG and 2 mg of DOX were dissolved in PBS buffer solution (pH 8.0). Then, CBAA monomer and TK-CL were added and dissolved in the DOX/ICG-BSA solution. After bubbling with nitrogen for 5 min, the aqueous solution was slowly added dropwise to the organic continuous phase. A stable microemulsion was formed after ultrasonic treatment. Subsequently, 10 μL of ammonium persulfate (APS) solution (20% w/v) was added to the emulsion. Five minutes later, polymerization was initiated by adding 8 μL of tetramethylethylenediamine (TEMED), followed by rapid stirring at 4 °C. After 2 h of reaction, the organic solvent was removed through vacuum distillation using a rotary evaporator, and the nanogels were precipitated. The resulting precipitate was washed three times with cold acetone and dried under vacuum for 30 min. The nanogels were then resuspended in PBS buffer solution (pH 7.4) and centrifuged to remove insoluble materials. The clear liquid was purified using an ultrafiltration tube with a molecular

weight cut-off (MWCO) of 100 kDa to remove free B-ICG and DOX.

The size and polydispersity index of nanogels were determined using dynamic light scattering (DLS). The yield of nanogels was calculated using the equation below: Yield (%) = $m/m_0 \times 100\%$, where m is the weight of lyophilized nanoparticles and m_0 is the total weight of the monomers and crosslinkers.

To evaluate the ROS-sensitive release capacity of different TK-CL-containing nanogels, 2 mL of DOX-encapsulated nanogel solution in pH 7.4 PBS buffer with 10 mM H_2O_2 was prepared. The solutions were transferred into dialysis bags with a molecular weight cut-off (MWCO) of 14 kDa. These bags were submerged in 40 mL of corresponding buffer and placed in an incubator shaker with gentle shaking (75 rpm) at 37 °C to mimic the in vivo environment. At predetermined time points, 3 mL of buffer was withdrawn from outside the dialysis bag to measure the amount of DOX using UV/Vis analysis, and 3 mL of fresh solution was added to replenish the volume. The amount of DOX was calculated according to the absorbance values at 480 nm, and the cumulative DOX release was presented as the total amount of released DOX during the incubation period.

Table S1. Composition and dosage in polymerization to evaluate optimal ratios of AOT and Brij-30.

$M_{AOT}/M_{Brij\text{-}30}$	**1/5**	**1/4**	**1/3**	**1/2**	**1/1**	**2/1**	**3/1**
AOT (g)	0.25	0.30	0.38	0.50	0.75	1.00	1.50
Brij 30 (g)	1.25	1.20	1.13	1.00	0.75	0.50	0.50
TK-CL (mg)	20.00						
CBAA (mg)	200.00						
PBS (mL)	0.60						
n-hexane (mL)	20.00						

Table S2. Composition and dosage in polymerization to evaluate optimal water-oil ratios.

V_{PBS}/V_{hexane}	**1/200**	**1/100**	**3/100**	**1/20**	**1/10**
AOT (g)	0.50				
Brij 30 (g)	1.00				
TK-CL (mg)	20.00				
CBAA (mg)	200.00				
PBS (mL)	0.10	0.20	0.60	1.00	2.00
n-hexane (mL)	20.00				

Table S3. Composition and dosage in polymerization to evaluate optimal ratios of crosslinker and monomer.

M_{TK-CL}/M_{CBAA}	1/2.5	1/5	1/10	1/25	1/50
AOT (g)	0.50				
Brij 30 (g)	1				
TK-CL (mg)	20				
CBAA (mg)	50	100	200	500	1000
PBS (mL)	0.60				
n-hexane (mL)	20				

Table S4. Composition and dosage in polymerization to evaluate optimal TK-CL contained nanogel.

M_{TK-CL}/M_{CBAA}	1/40	1/20	1/10	1/5	1/2
AOT (g)	0.5				
Brij 30 (g)	1.0				
TK-CL (mg)	5	10	20	40	100
CBAA (mg)	200				
PBS (mL)	0.60				
n-hexane (mL)	20				

Characterization

^{1}H-NMR spectra were obtained using a Varian 400 MHz spectrometer with $CDCl_3$, D_2O and DMSO d_6 as the solvenst at ambient temperature. FT-IR spectra of the compounds were recorded on a Nicolet/Nexus 670. The Size and zeta potentials of nanogels were measured via dynamic light scattering (DLS). Prior to analysis, samples were filtered through a 450 nm filter. DLS measurements were performed at 25 °C using a 90 Plus/BI-MAS equipment (Malvern Panalytical, Malvern, United Kingdom), and data were collected with an autocorrelator with a 90° detection angle of scattered light. The results are presented as the mean ± standard deviation (SD) of three independent measurements. Transmission electron microscopy (TEM) images of nanogels were captured using a Philips CM120 transmission electron microscope (Philips, Eindhoven, the Netherlands) at an accelerating voltage of 80 kV. A 10 μL drop of the sample solution was placed on a copper grid coated with amorphous carbon, stained with 15 μL of 3 wt% uranyl acetate solution for 1 min, and excess stain was removed with filter paper. The grid was then dried overnight in a desiccator before TEM observation.

Cell culture

MDA-MB-231 human breast cancer cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were kept in a humidified incubator at 37 °C with 5% CO_2 and 90% humidity.

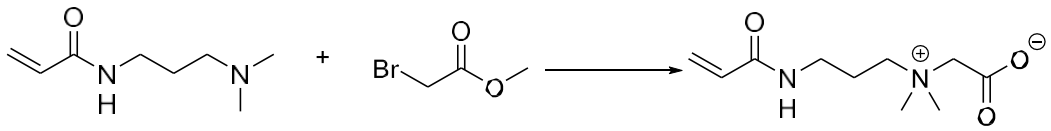

Figure S1. Synthesis route of CBAA-based monomer.

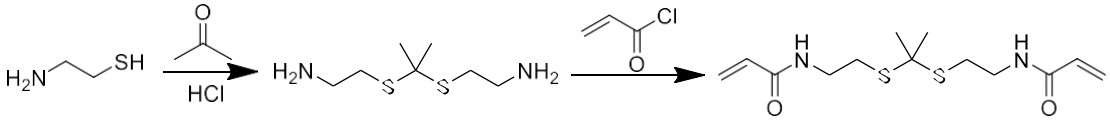

Figure S2. Synthesis route of thioketal (TK)-containing crosslinker.

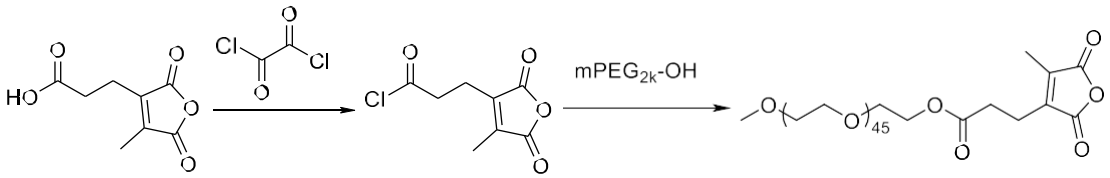

Figure S3. Synthesis rout of poly(ethylene glycol)-carboxydimethyl maleate (PEG-CDM) polymer.

Figure S4. Synthetic route of poly(ethylene glycol)-carboxydimethyl maleate-cell penetrating peptide (PEG-CDM-CPP).

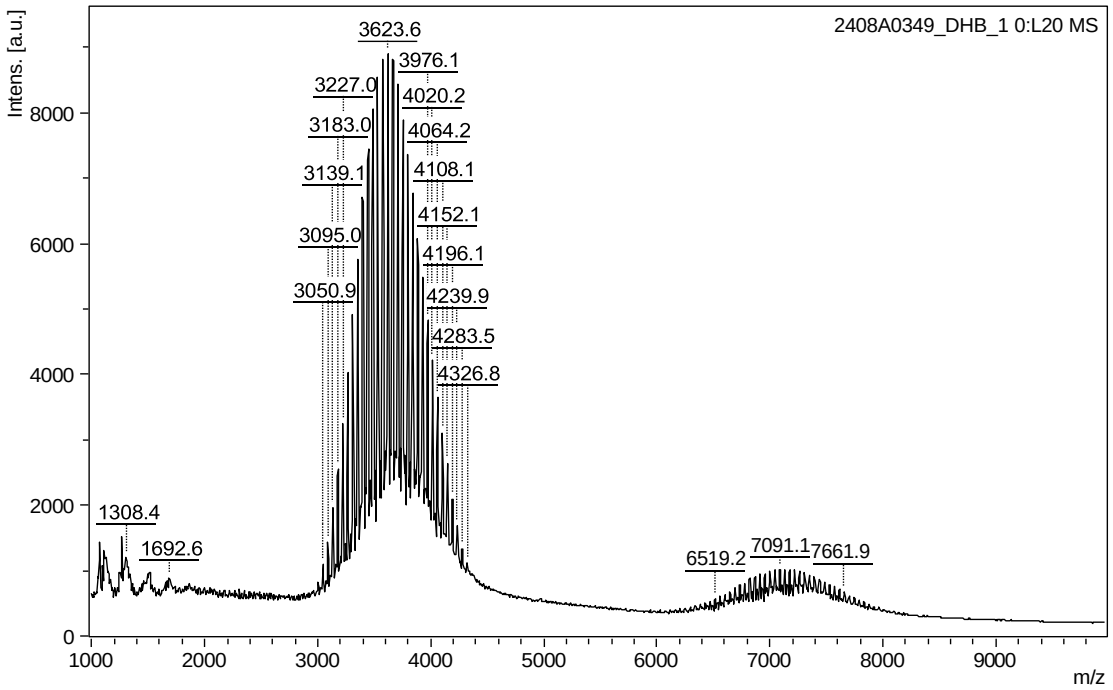

Figure S5. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of PEG-CDM-CPP (M: 3623.6, Calculated: 3625).

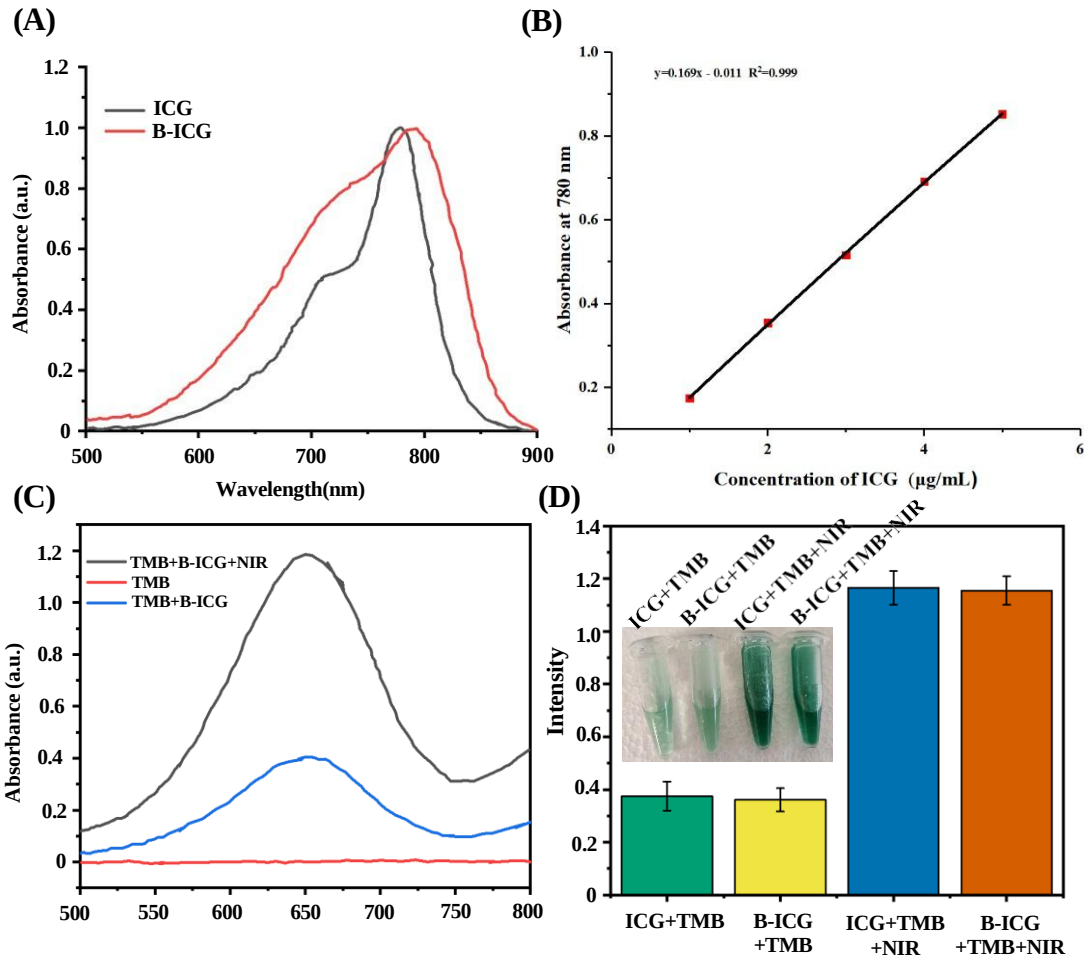

Figure S6. Comparative analysis of ICG and ICG conjugated BSA (B-ICG) in PBS solution. (A) Normalized absorption spectra of the ICG and B-ICG. (B) Standard curve of ICG, plotted as absorbance at 780 nm against ICG concentration. (C) Normalized absorption spectra of the 3,3',5,5'-tetramethylbenzidine (TMB), TMB+B-ICG without NIR irradiation, and TMB+B-ICG with NIR irradiation in PBS. TMB undergoes oxidation in the presence of ROS, producing a blue-colored product that can be quantified spectrophotometrically. (D) Optical images and UV-Vis absorption intensity to evaluate ROS-generation from B-ICG versus free ICG under NIR exposure.

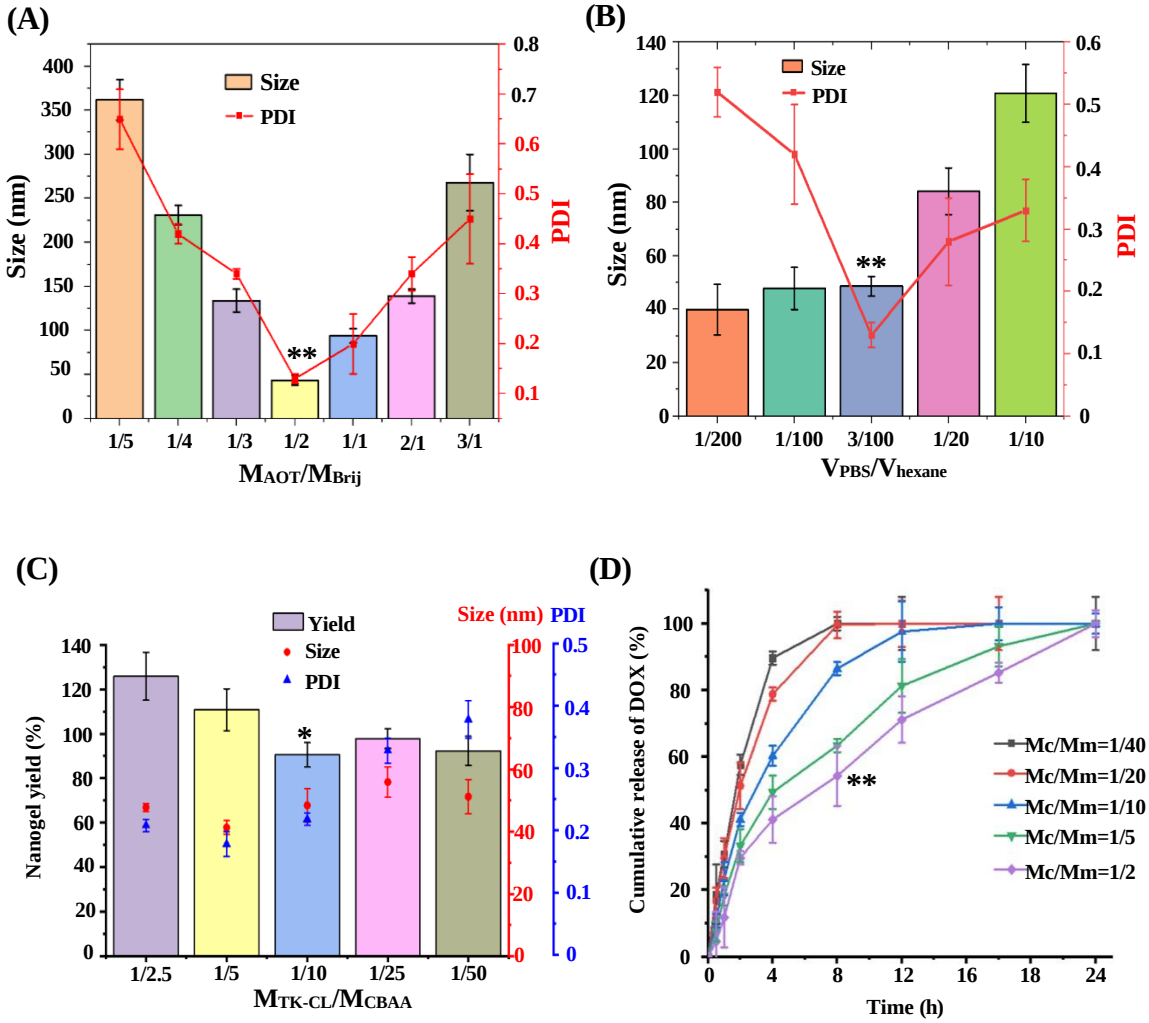

Figure S7. Optimization of polymerization conditions. (A) Particle size and stability at different ratios of AOT to Brij-30. ** indicates $p < 0.01$ compared to other groups in both size and PDI. (B) Changes in nanogel size and polydispersity index (PDI) at varying water-oil ratios. ** indicates $p < 0.01$ compared to 1/2 and 1/10 groups in both size and PDI. (C) Effects of different crosslinker-to-monomer ratios on nanogel yield, size, and PDI. * indicates $p < 0.05$ compared to 1/2.5 and 1/5 groups in nanogel yield, size, and PDI. (D) Cumulative release of DOX from nanogels at different TK-CL dosages. M_c/M_m represents $M_{crosslinker}/M_{monomer}$. ** indicates $p < 0.01$ compared to M_c/M_m of 1/40, M_c/M_m of 1/20, and M_c/M_m of 1/10 groups in cumulative release of DOX.

(A)

Loading efficiency and content

Composition	ICG	Dox
Loading content	2.32%	2.81%
Loading efficiency	32.1%	35.2%

(B)

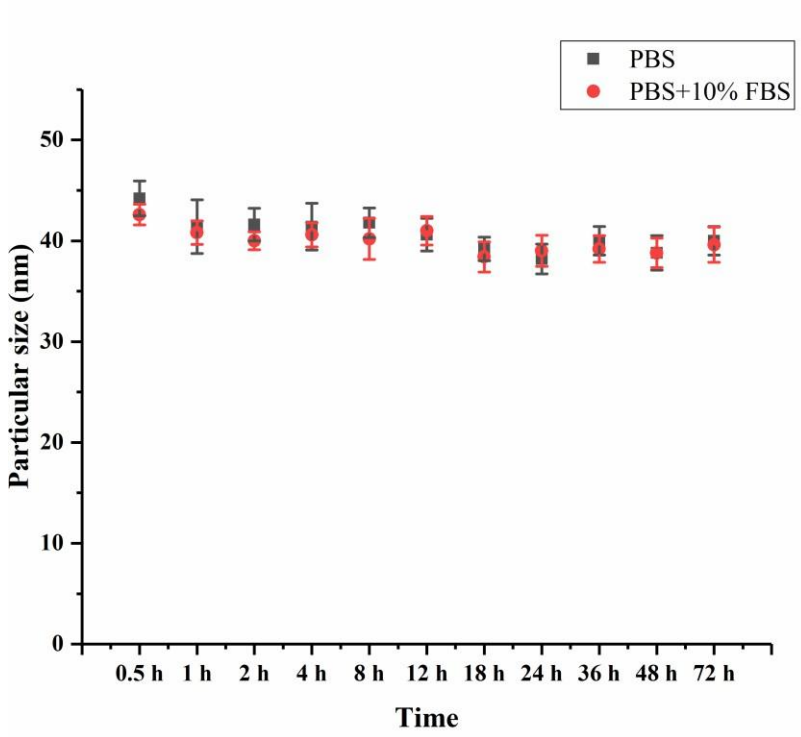

Figure S8. (A) Loading efficiencies and contents of DOX and ICG. (B) Colloidal stability of PCP-NC@D&I in PBS (pH 7.4) containing 10% FBS using dynamic light scattering (DLS) analysis.

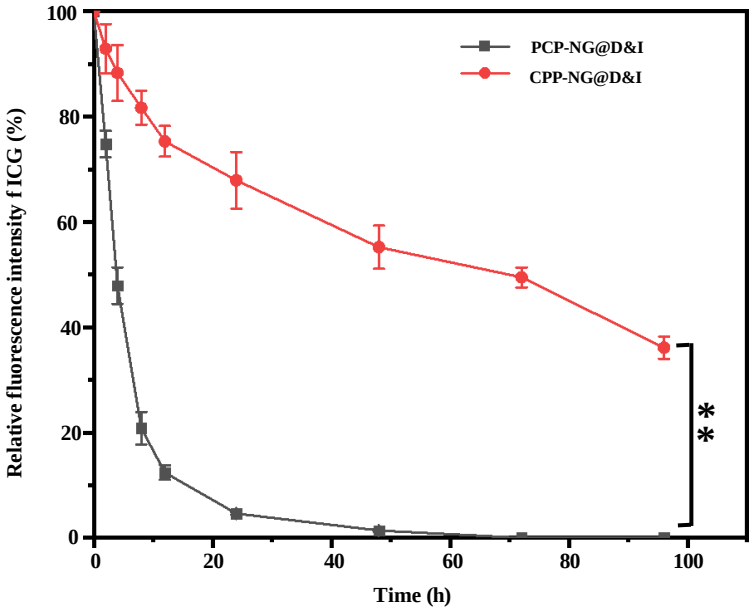

Figure S9. Blood circulation time of PCP-NG@D&I and CPP-NG@D&I in Sprague Dawley (SD) rats. The blood circulation time of PCP-NG@D&I and CPP-NG@D&I was monitored in SD rats, highlighting the sustained circulation of PCP-NG@D&I compared to CPP-NG@D&I.

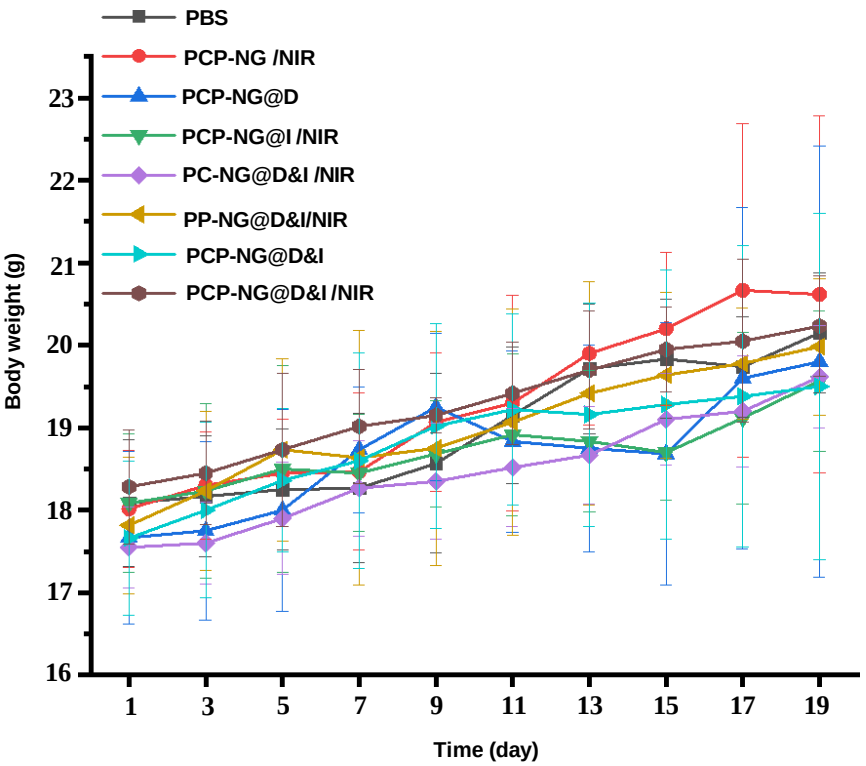

Figure S10. Body weights of tumor-bearing animals receiving different treatments.

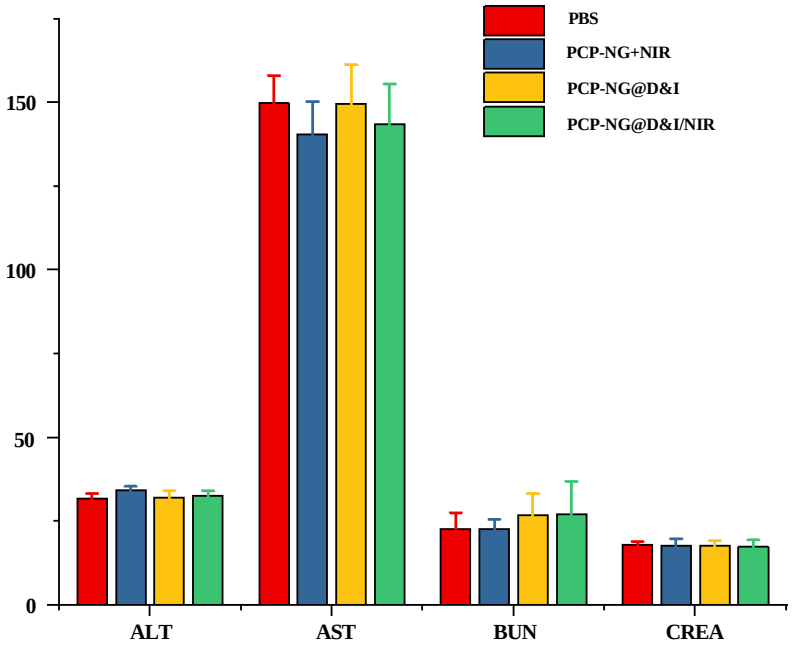

Figure S11. Evaluation of liver and renal functions in healthy BALB/c mice after various treatments. Blood biochemistry indicators, including aspartate aminotransferase (AST, U/L) and alanine aminotransferase (ALT, U/L) for liver function and blood urea nitrogen (BUN, mg/dL) and crea (CREA, μmol/L) for renal function, were assessed in healthy BALB/c mice following different treatments to evaluate potential systemic toxicity.