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Supporting Information

A Facile Combined Therapy of Chemotherapeutic Agent and MicroRNA for Hepatocellular Carcinoma Using Non-Cationic Nanogel†

Dingyue Zhang,‡[a] Mingxiang Zuo,‡[b] Jinhui Zhou,‡[a] Siyu Ouyang,[a] Shuang Liu,[b] Jianming Yuan,[b] Chiyi Ou,[b] Qinghua Chen,[b] Dongsheng Yu,[b] Du Cheng,*[b] Jin Wang*[a]

[a.]Department of Radiology, the Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510630, P.R. China

E-mail :wangjin3@mail.sysu.edu.cn

[b.]PCFM Lab of Ministry of Education, School of Material Science and Engineering, Sun Yat-Sen University, Guangzhou 510275, P.R. China

E-mail :chengdu@mail.sysu.edu.cn

Synthesis of CTSB crosslinker (CSB-CL)

The synthesis route of cathepsin B enzyme-sensitive cross linker (CSB-CL) was illustrated in **Figure S1**. The procedure was referred to our previously published work [1]: 0.20 g of peptide with the sequence of Ac-GLKGFLGKLG-NH_2 and 0.20 g of methacryloyl chloride were added to a 50 mL bottle and dissolved in 20 mL of DMSO. The solution was stirred at room temperature overnight, then transferred to a dialysis bag with a molecular weight cutoff (MWCO) of 0.5 kDa. It was dialyzed in methanol for 1 d, followed by another day in deionized water for, and finally lyophilized to obtain 0.21 g of white powder (yielding: 93.6 %).

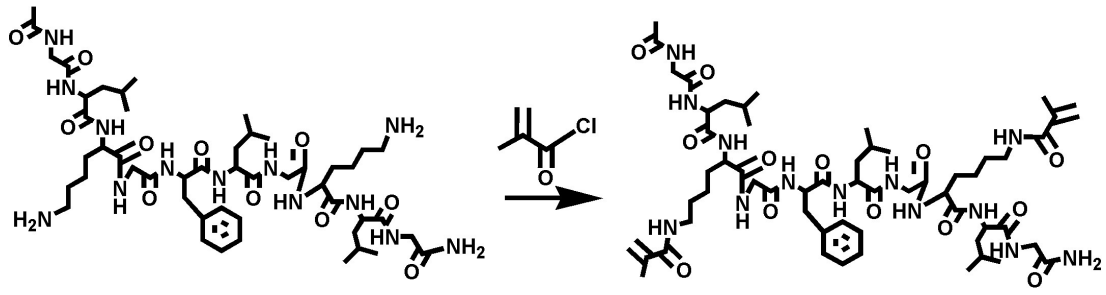

Figure S1. Synthesis route of crosslinker CSB-CL.

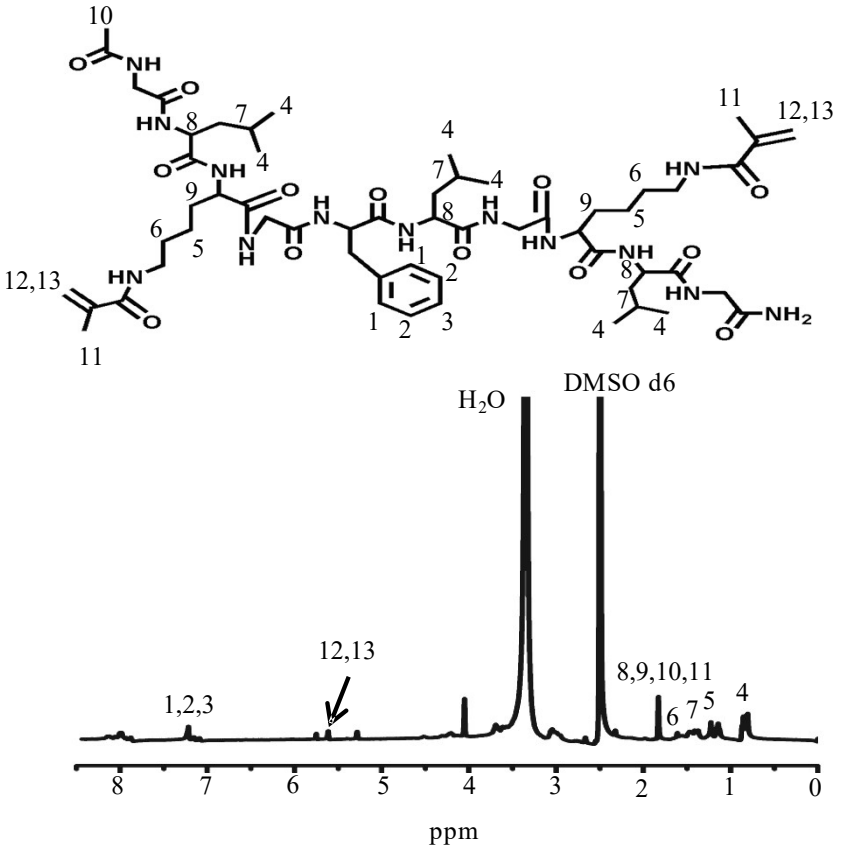

Figure S2. [1]H-NMR spectrum of crosslinker CSB-CL.

Synthesis of CBAA monomer

The procedure was referred to our previously published work [1]. Briefly, 6.24 g of N, N-dimethylaminopropyl acrylamide (40 mmol), 9.18 g of methyl bromoacetate (60 mmol) and 25 mL of dichloromethane were added to a 100 mL flask and stirred at room temperature for 8 h under nitrogen atmosphere. The precipitate was collected and washed with anhydrous acetone of 500 mL. The residual solvent was removed through vacuum drying. Afterwards, the mentioned product was dissolved in 0.05 M of sodium hydroxide solution and kept stirring for 3 h. Then the water was removed via reduced pressure rotating evaporation and the product was washed with anhydrous acetone and anhydrous ether. Finally, the product was dried under vacuum and white powder was obtained (8.68 g, yielding: 94.8%).

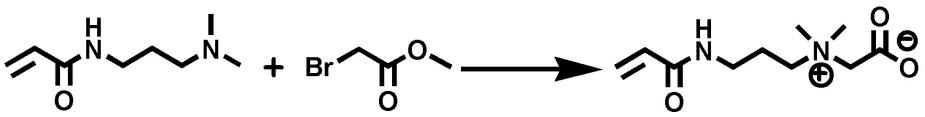

Figure S3. Synthesis route of monomer CBAA.

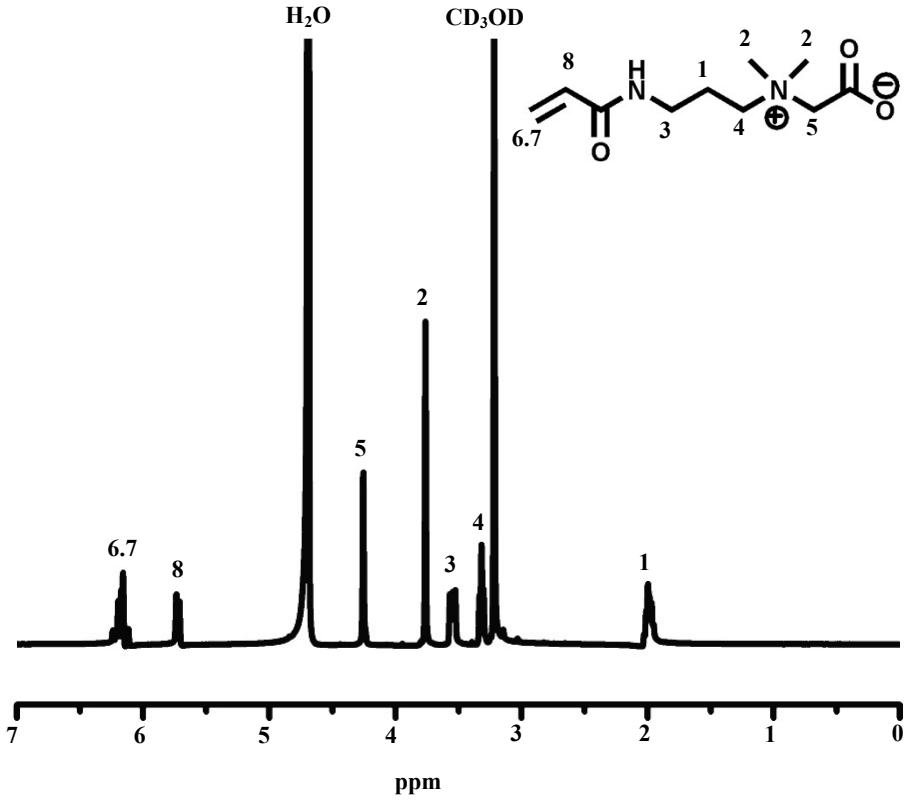

Figure S4. [1]H-NMR spectrum of monomer CBAA.

Synthesis of PDPA-PCB-Dimethylacetyl-methacrylamine (CBPD) monomer

A mixture of 0.75 g (2.06 mmol) of 1-dodecyl-(dimethylacetic acid) trithiocarbonate (DDAT), 1.75 g (8.24 mmol) of 2-diisopropylaminoethyl methacrylate, 33.74 mg (0.206 mmol) of 2, 2'-azodiisobutyronitrile were dissolved in 60 mL of a mixed solvent of reaction grade DMF and water (95:5, v/v). After nitrogen protection for 30 min at room temperature, the reaction mixture was heated in an oil bath at 70 °C and stirred for 24 h. Then, 0.98 g (6.18 mmol) of 3- [(3-acrylamidopropyl) dimethylammonium] acetate was added, and reaction was performed overnight. The reaction system was then quickly cooled to room temperature. Afterwards, 6.77 g (41.13 mmol) of AIBN was added with nitrogen protection for 45 min, then the mixture was again heated to 70 °C for 24 h. The operation was repeated three times to fully remove the thiocarbonate group from the polymer. The reaction mixture was then precipitated three times in anhydrous diethylether, and the precipitate was washed with acetone and dried under vacuum. Consequently, 1.75 g of white solid was obtained.

Next, 1.5 g of obtained solid precipitate, 0.107 g of 3-methylacrylamine, 0.216 g of N-hydroxysuccinimide (NHS, 1.88 mmol), 0.387 g of N, N-dicyclohexyl carbodiimide (DCC,1.88 mmol) were dissolved in 30 mL of DMSO and the reaction was allowed to proceed at room temperature for 1 d. The precipitate product of 1,3-dicyclohexylurea (DCU)formed during the reaction was removed using an oil filter membrane. The resulting solution was precipitated in anhydrous, centrifuged, washed for three times, and dried under vacuum overnight. Finally, 1.40 g of yellow solid was obtained.

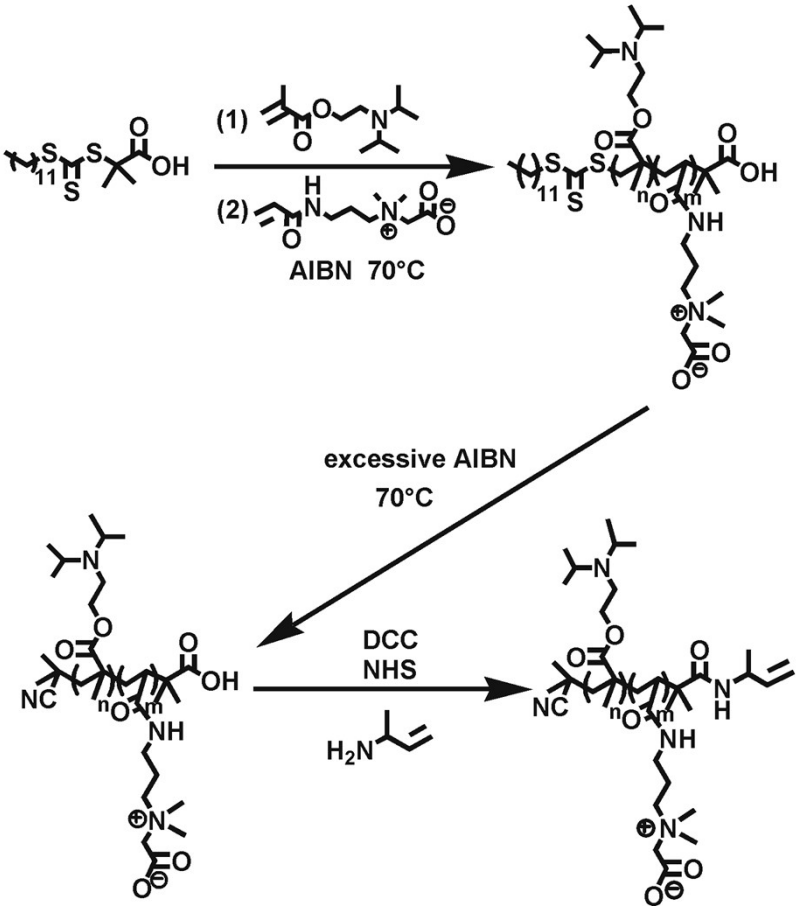

Figure S5. The diagram of synthesis route of CBPD.

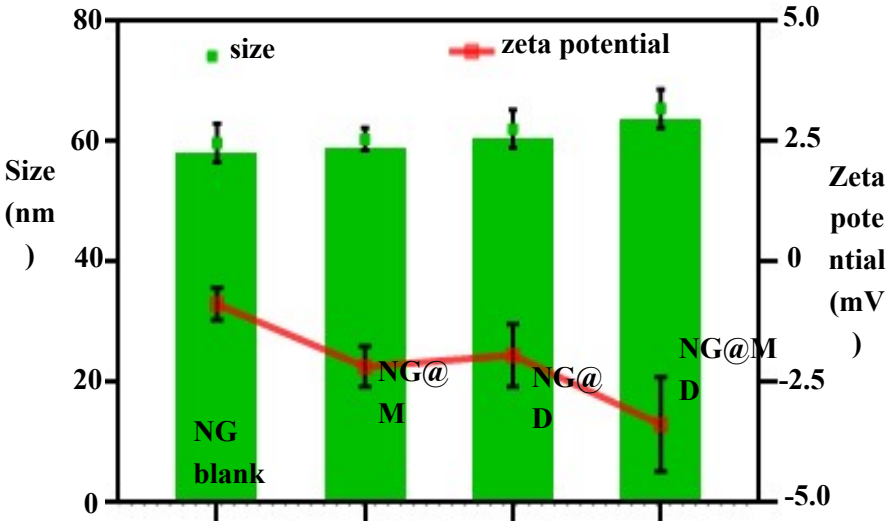

Figure S6. Particle size and zeta potential of nanogels (the ratio among CBAA, CSB-CL and CBPD was 60:10:4, n=3).

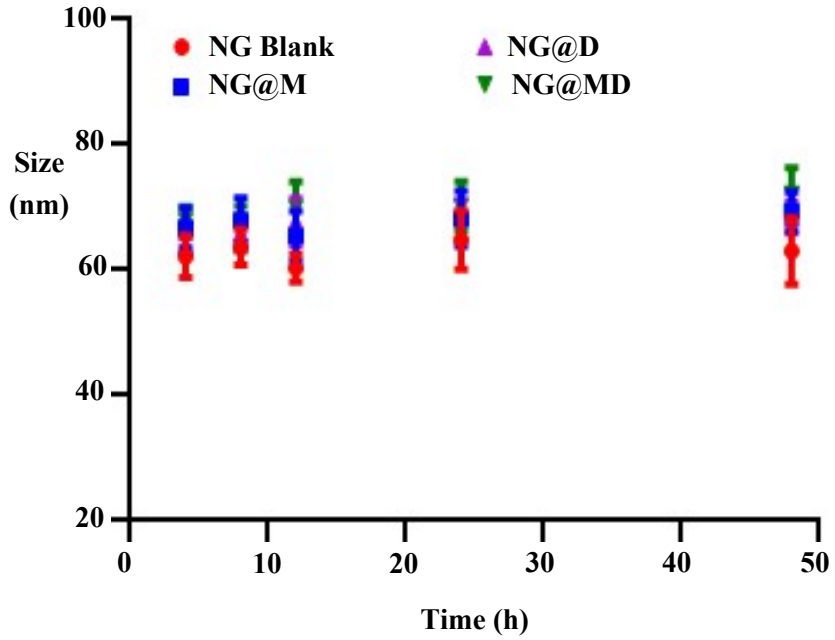

Figure S7. Stability of nanogels in PBS of pH 7.4 containing 10% FBS measured by dynamic light scattering, n=3.

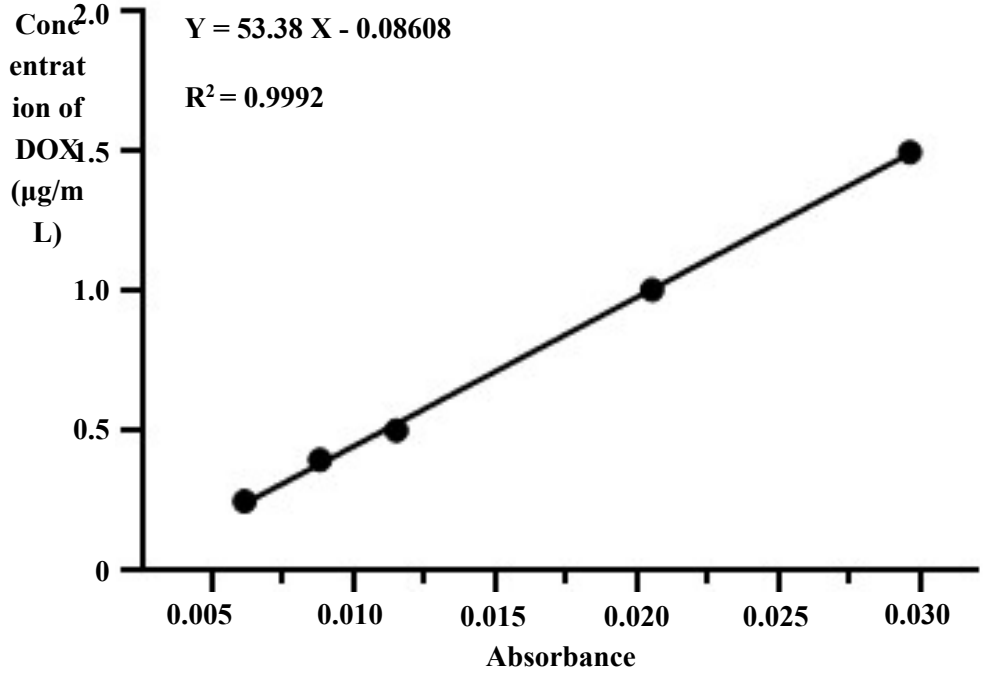

Figure S8. Standard curve of DOX concentration against absorbance at 488 nm.

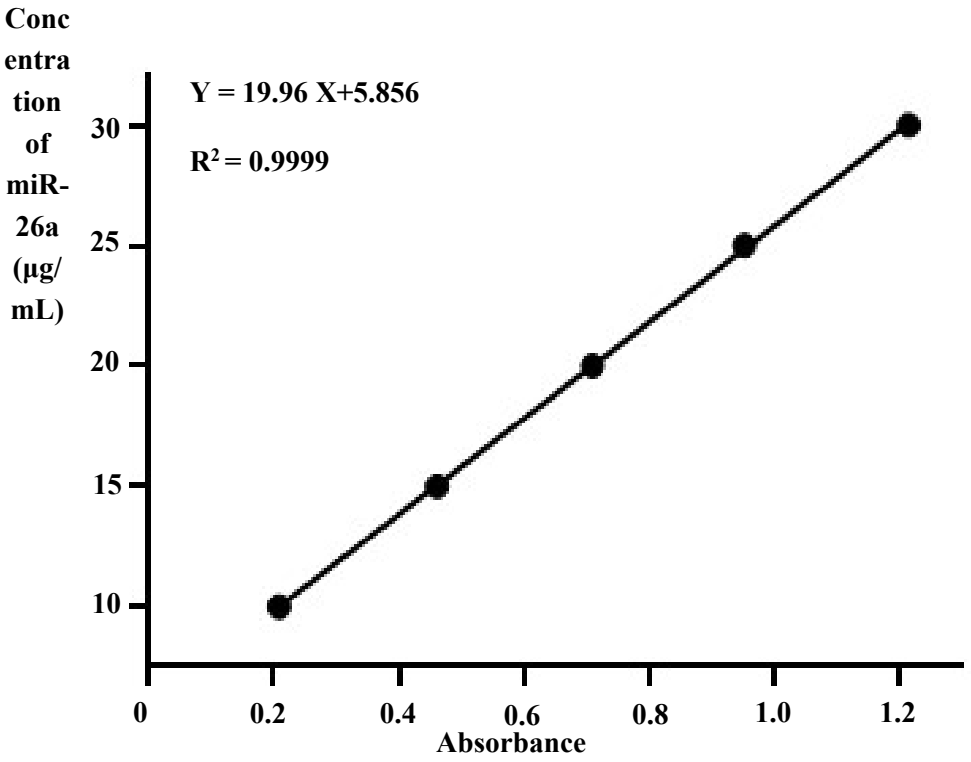

Figure S9. Standard curve of miR-26a concentration against absorbance at 260 nm.

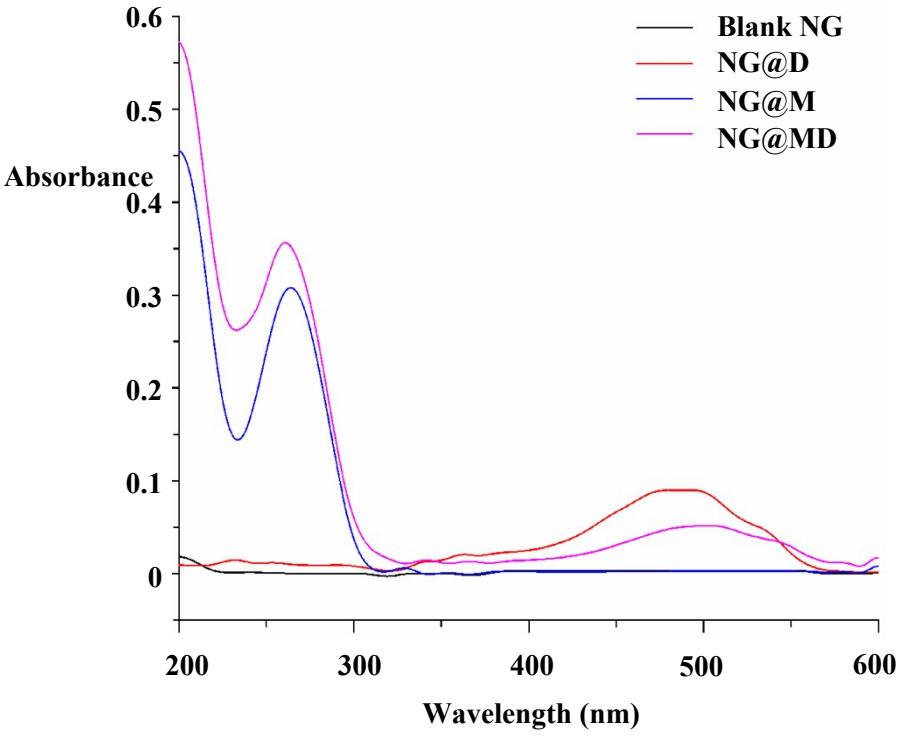

Figure S10. The UV-absorbance spectra of Blank NG, NG@D, NG@M and NG@MD. The absorbance peaks of DOX and miRNA at 488nm and 260nm were completely separated.

Degradation of nanogel

3 mL of blank nanogel (1 mg/mL) incubated with 1 mM of papain at pH 5.0 was placed in the dialysis bag with a MWCO of 14 kDa and submerged in 6 mL of buffer at pH 5.0 for 12 h and 24 h. At each preset time intervals, solution outside the dialysis bag was collected and analyzed by MALDI-TOF mass spectrometry.

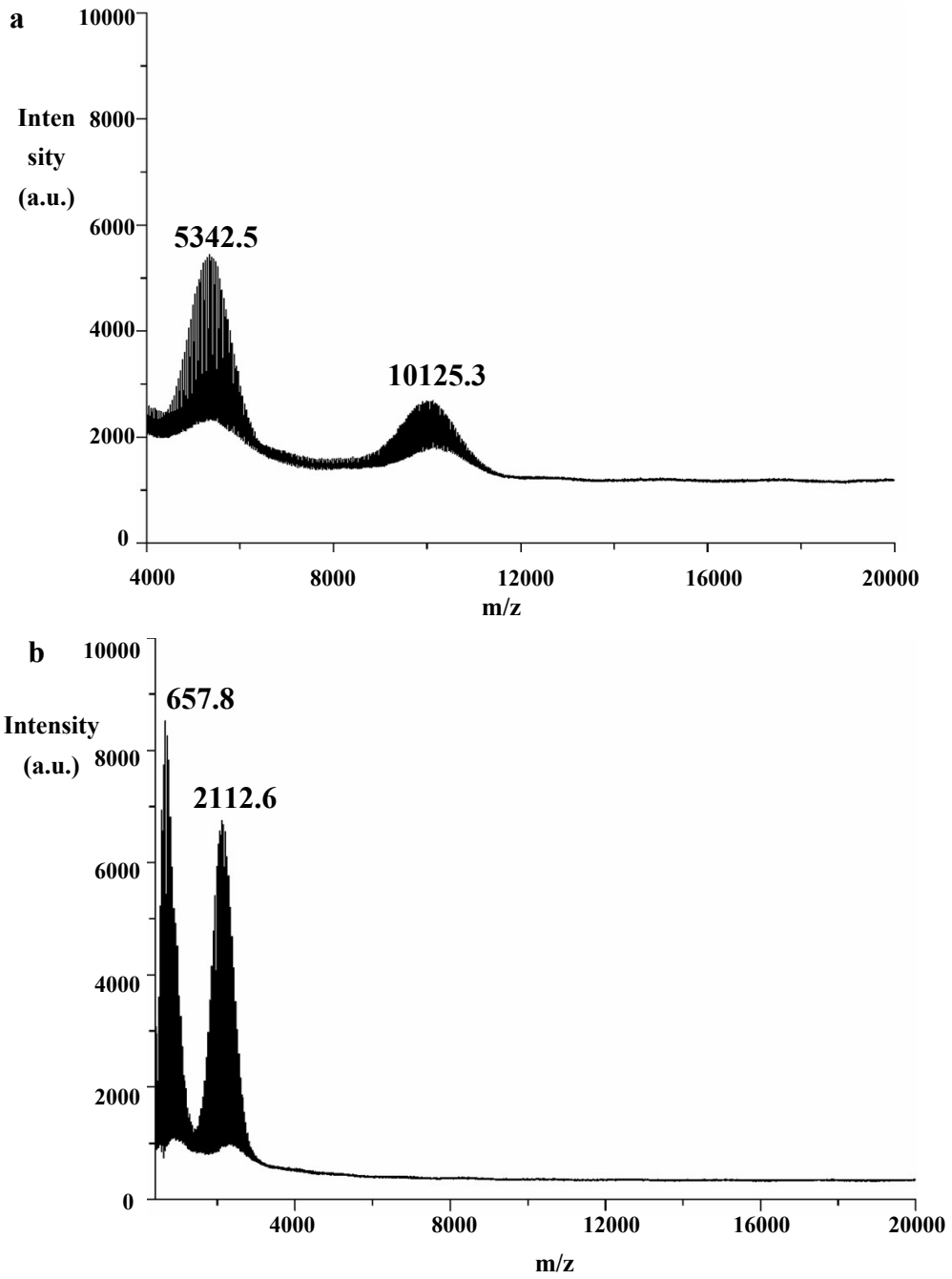

Figure S11. MALDI-TOF mass spectra of nanogel incubated with 1 mM at pH 5.0 for (a) 12 h, (b) 24 h.

Cell Culture

Hepatoma cells were cultured in high glucose DMEM containing 10% serum fetal bovine serum (FBS) and placed in an incubator with 5% CO2 at 37 °C. In addition, normal hepatic parenchymal THLE-2 cells were cultured in BEGM containing 5 ng/mL Epidermal Growth Factor (EGF) and 10% FBS. When the cells reached confluence of 80-90%, they were trypsinized for subsequent use.

In Vitro Cytotoxicity Assay

The assessment of cell cytotoxicity was evaluated using the CCK-8 assay and live/dead cell staining method. In the CCK-8 assay, SK-Hep1 cells were seeded in 96-well plates at 1×10^4 cells per well. After 12 h, the nanogels containing different concentrations of drugs were added and cultured for 36 h. Subsequently, the viability of the SK-Hep1 cells was determined by CCK8 assay.

For the live/dead cell staining, SK-Hep1 cells were seeded in confocal dishes overnight. They were then incubated with nanogels containing various concentrations of the drug for 36 h. Then, the cells were stained with calcein-AM and DAPI for 30 min at 37 °C. Finally, the cells were visualized by CLSM.

RT-qPCR Assay and Western Blot Assay

The protocols for extracting total RNA and preparing complementary DNA were based on previous publication [2]. For the miRNA expression assay, the relative level of miR-26a was normalized to U6 (Accurate Biology, AG11745, China). Quantitative mRNA expression was determined using the Fast Start Universal SYBR Green Master real-time PCR system (Step One Plus, ABI) [2]. Primers sequences are detailed in Supplementary **Table S2.**

Proteins obtained from SK-Hep1 cells treated with different nanogels, the methods were referred to previous published work [3]. The antibodies included anti-USP9X (1:2000; Abcam, Cambridge, UK), anti-LOXL2 (1:2000; Abcam, Cambridge, UK), anti-CDK8 (1:1000; Proteintech, Wuhan, China), anti-PAK2 (1:2000; Abcam,

Cambridge, UK) and anti-GAPDH (1:10000; Abcam, Cambridge, UK). The gray value assay was performed with ImageJ software.

Cell Migration and Invasion Assay

Cell migration and invasion capabilities were assessed using Transwell chambers. For the migration assay, SK-Hep1 cells (5×10^4) were suspended into the upper chamber without serum or growth factors. The lower chamber supplemented with medium containing 10% FBS as a chemoattractant. At 24 h of incubation with different nanogels, the cells that had migrated to the lower chamber were fixed with 4% paraformaldehyde, then stained with a 0.1% crystal violet solution, washed with PBS, dried, and counted in five fields at random. For the invasion assay, SK-Hep1 cells (5×10^4) were planted into the upper chamber and incubated for 48 h, the cells were then stained and photographed as above.

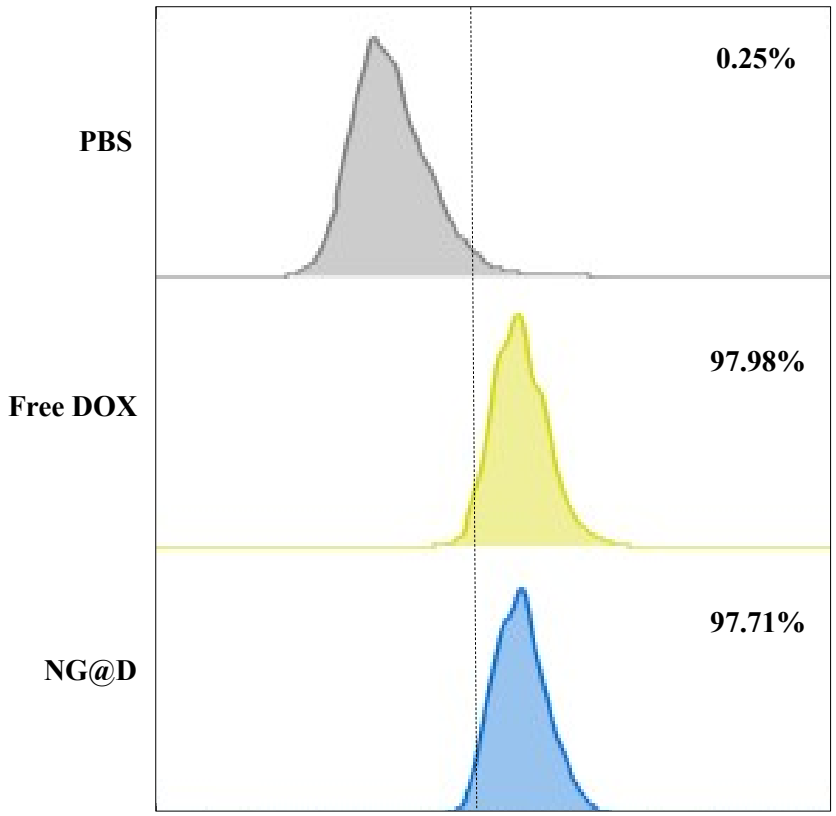

Figure S12. Cell uptake efficiency of free DOX and NG@D was analyzed by flow cytometry analysis, n = 3.

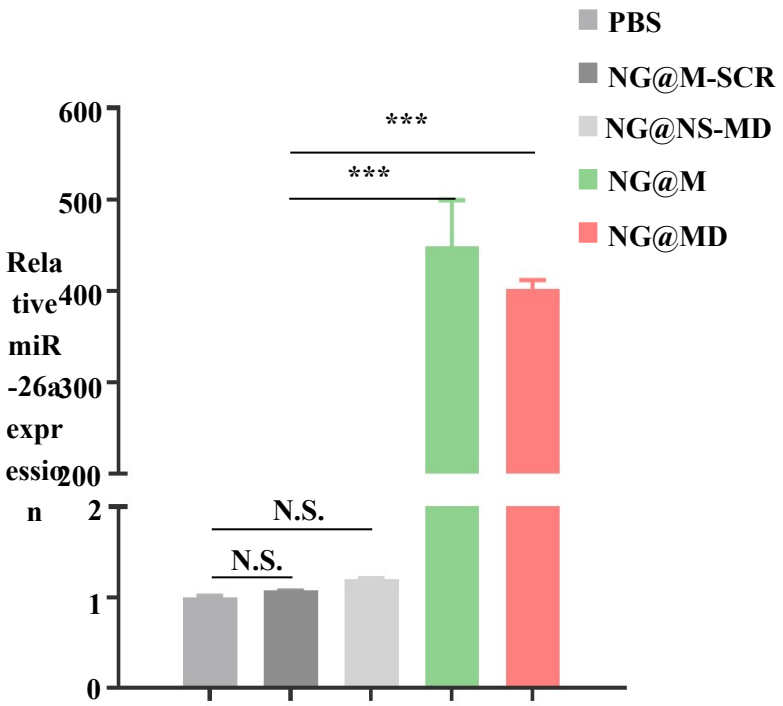

Figure S13. Expression of miR-26a after treatment with different nanogels. Data are presented as the mean ± SD. (n = 3, ***p < 0.001)

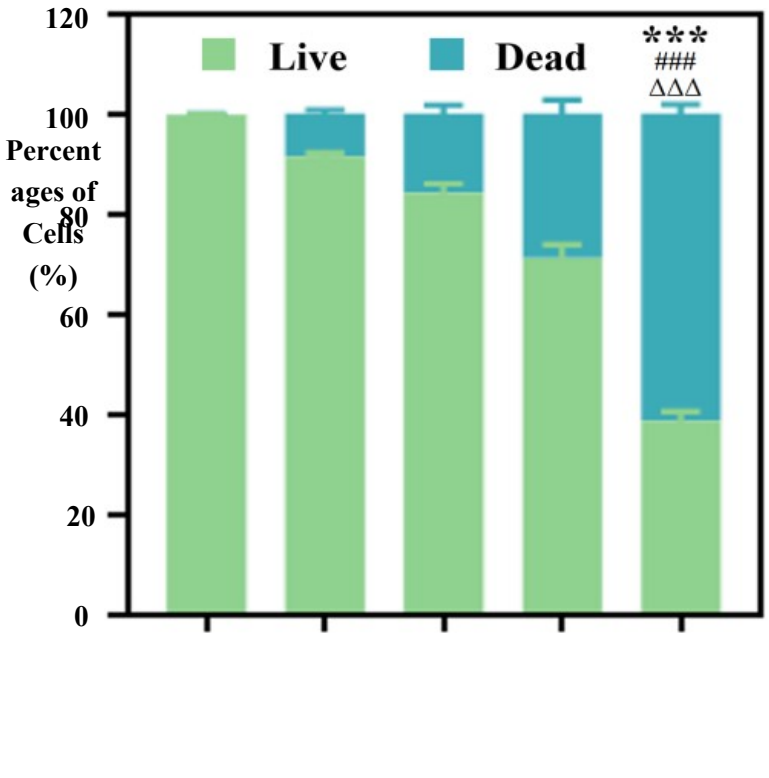

Figure S14. Quantitative statistical analysis of live/dead staining was performed using Image J. software. ***p < 0.001 vs PBS; ###p < 0.001 vs NG@M; ΔΔΔp < 0.001 vs NG@D, n = 3.

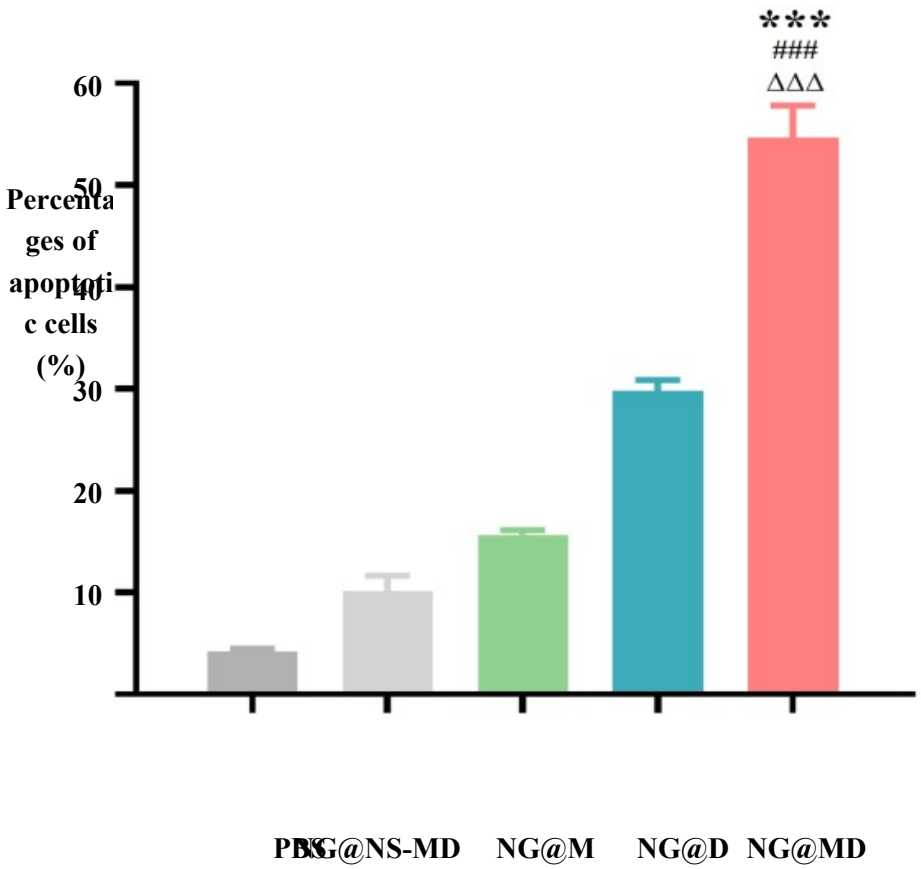

Figure S15. Quantitative analysis of apoptotic SK-Hep1 cells. The percentage of apoptotic cells were calculated according to the upper right and lower right quadrants (A2+A4) over the total cell numbers (A1 + A2+ A3 + A4). ***$p < 0.001$ vs PBS; ###$p < 0.001$ vs NG@M; ΔΔΔ$p < 0.001$ vs NG@D, n = 3.

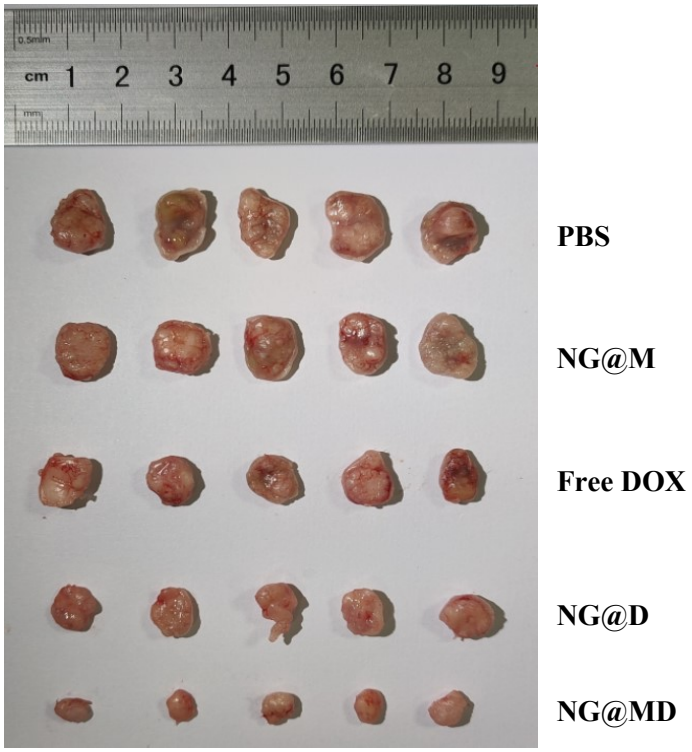

Figure S16. Typical images of subcutaneous tumors collected from different groups after 21 d treatment.

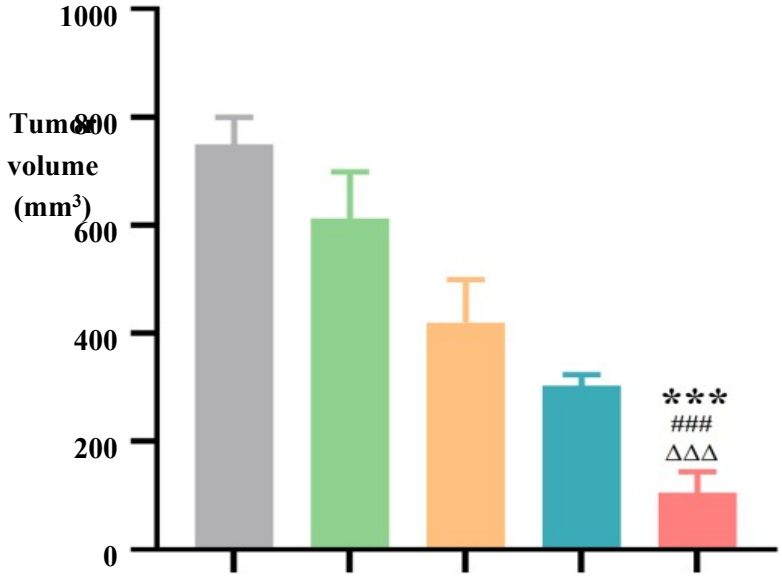

Figure S17. Tumor volume of mice in different groups at 21 d (n = 5, mean ± SD; ***p < 0.001 vs PBS; ###p < 0.001 vs NG@M; ΔΔΔp < 0.001 vs NG@D).

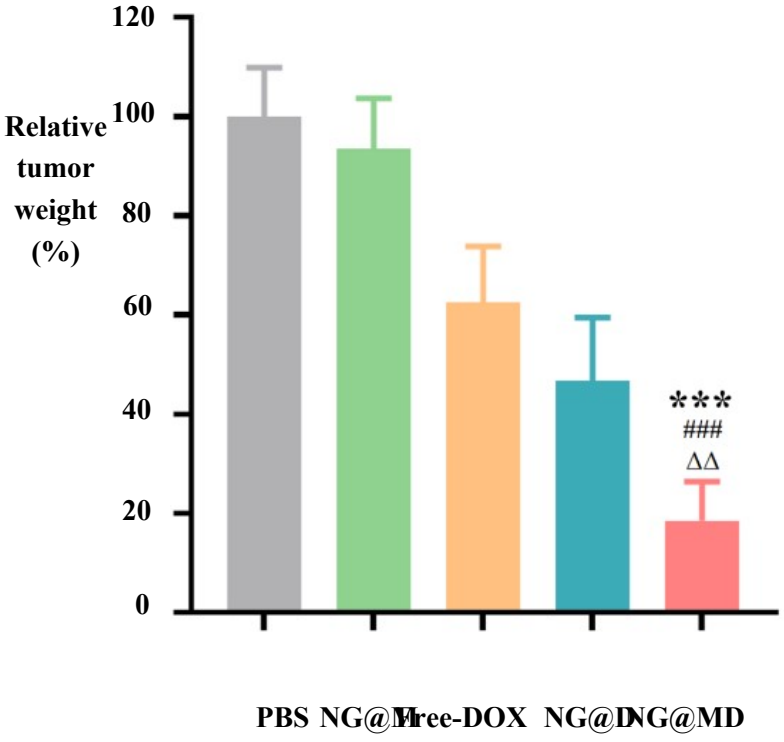

Figure S18. The average tumor weights for each group (n=5, mean ± SD; ***p < 0.001 vs PBS; ###p < 0.001 vs NG@M; ΔΔp < 0.01 vs NG@D).

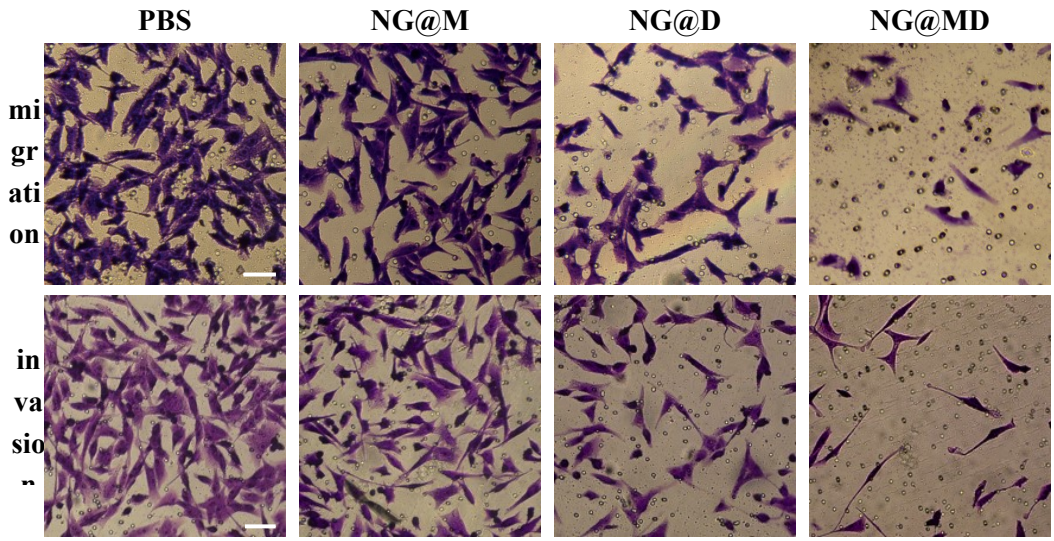

Figure S19. Migration and invasion abilities of SK-Hep1 cells treated with different nanogels determined through Transwell analysis. Scale bars, 50 μm. DOX concentration if applied: 0.4 μg/mL, miR-26a concentration if applied:100 nM.

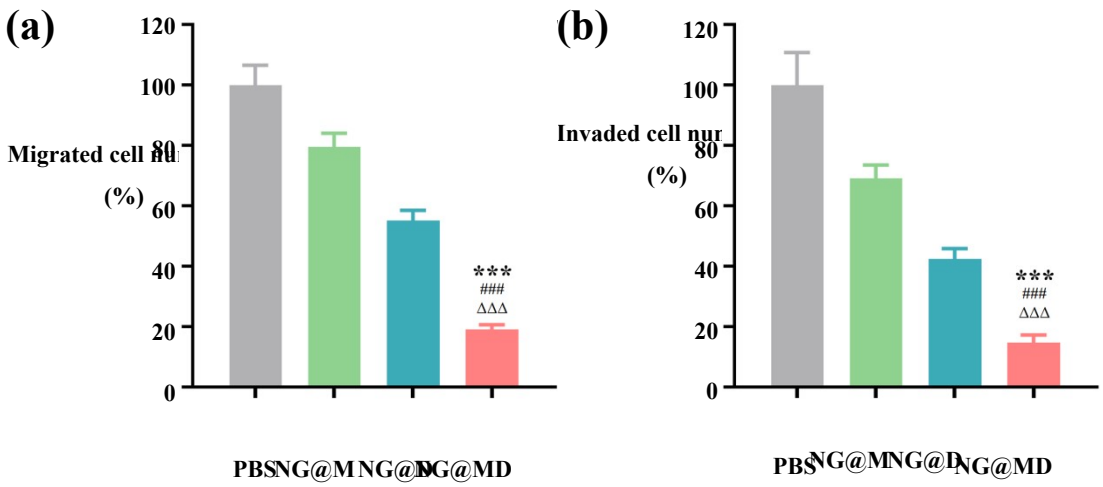

Figure S20. Quantitative analysis of migrated and invaded cell numbers using Image J software. The migrated and invaded cells in five random microscopic fields were counted. ***p < 0.001 vs PBS; ###p < 0.001 vs NG-miR-26a; ΔΔΔp < 0.001 vs NG@D.

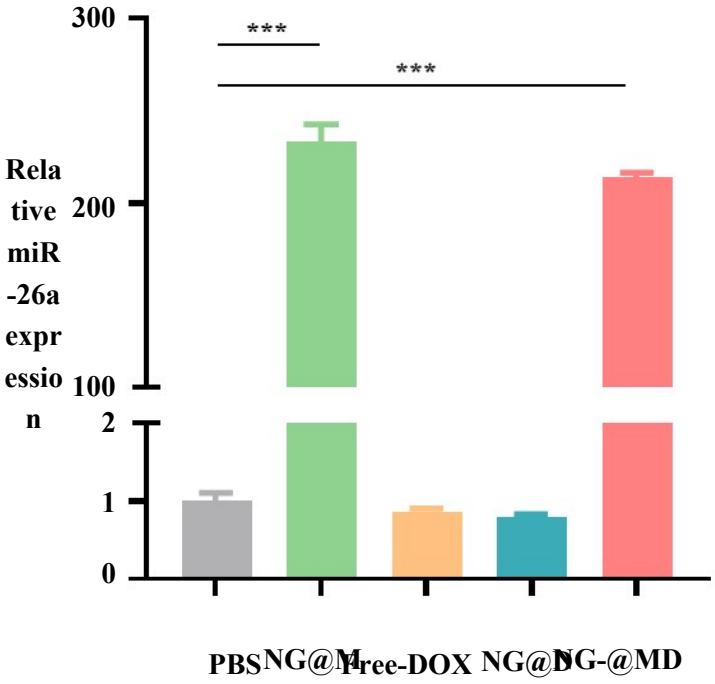

Figure S21. Expression of miR-26a in tumor after different treatment. Data are presented as the mean ± SD. (n = 3, ***p < 0.001)

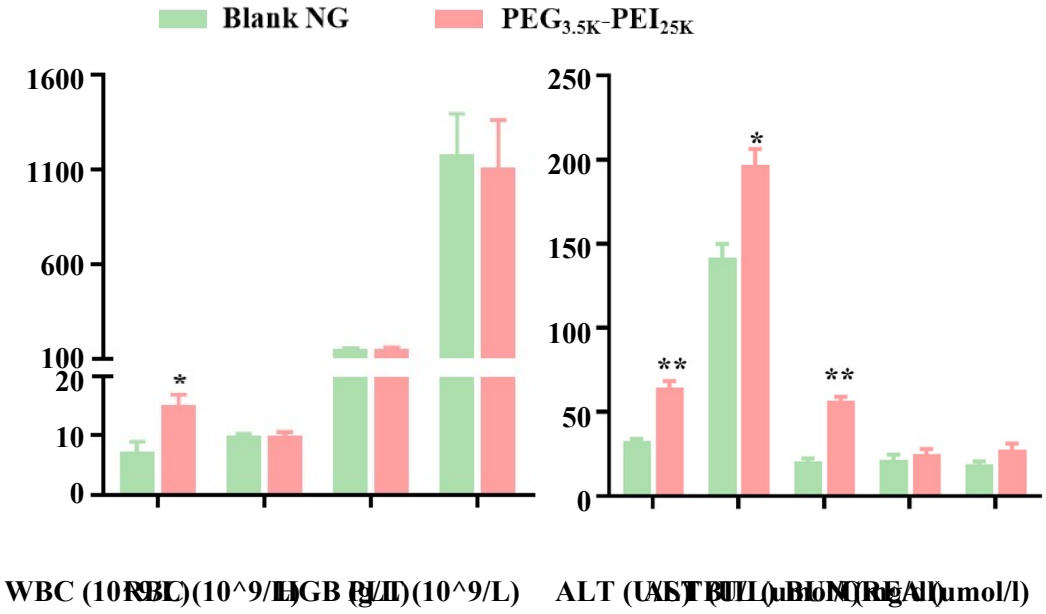

Figure S22. Blood routine examination and serum biochemical indicators analyses in animals. (n = 3, *p < 0.05, **p < 0.01)

Table S1. IC50 values of nanogel drugs for SK-Hep1 cells.

Incubation time	IC_{50} (μg/ml)		
	NG@D	NG@M-SCR/D	NG@MD
36 h	0.6229	0.5856	0.1859

$IC_{50,}$ half maximal inhibitory concentration.

Table S2. Primers used for qRT-PCR analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	CACCCACTCCTCCACCTTTGAC	GTCCACCACCCTGTTGCTGTAG
USP9X	GCCAGGTGTAGAAGGTGTGAATC	CGGATGATGGATTAGGAATGCTCTG
LOXL2	CTATGACCTGCTGAACCTCAATGG	TTGGCACACTCGTAATTCTTCTGG
PAK2	GTTCTACGACTCCAACACAGTGAAG	TTCATCATCATCCTCCTCCTCTGTC
CDK8	CAGCAGCAGCAGGGCAATAAC	TGAGGTAGTGGTAGGAGGAACAAC
miR-26a	GCTCGTTCAAGTAATCCAGG	AGTGCAGGGTCCGAGGTATT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

References

1. X. M. Zeng, M. X. Zuo, J. M. Yuan, G. Chen, S. Liu, C. Y. Ou, Q. H. Chen, C. Q. Wei, D. S. Yu and D. Cheng, *Advanced Functional Materials*, 2024, DOI: 10.1002/adfm.202406122.

2. L. J. Hu, S. C. Huang, G. J. Chen, B. Li, T. Li, M. Z. Lin, Y. Q. Huang, Z. C. Xiao, X. T. Shuai and Z. Z. Su, *Acs Applied Materials & Interfaces*, 2022, **14,** 31625-31633.

3. S. H. Deng, X. X. Li, S. Liu, J. F. Chen, M. Q. Li, S. Y. A. Chew, K. Leong and D. Cheng, *Science Advances*, 2020, **6.**