Supporting Information

Tumor microenvironment-activatable boolean logic supramolecular nanotheranostics based on pillar[6]arene for tumor hypoxia imaging and multimodal synergistic therapy

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1. General information

Glucose oxidase (GOx, 100 units/mg protein), porcine liver esterase (PLE, 15 units/mg) were purchased from Sigma-Aldrich. All reactions were performed in air atmosphere unless otherwise stated. The commercially available reagents were used as supplied without further purification. Chloroform and acetonitrile were dried through procedures described in the literatures, and other solvents were used without further purification unless otherwise stated. Column chromatography was performed with silica gel (200 – 300 mesh) produced by Qingdao Marine Chemical Factory, Qingdao (China). All yields were given as isolated yields. NMR spectra were carried out on a Bruker DPX 400 MHz spectrometer, using internal standard tetramethylsilane (TMS) and solvent signals as internal references at 298 K, and the chemical shifts (δ) were expressed in ppm and J values were given in Hz. Low-resolution electrospray ionization mass spectra (LR-ESI-MS) were acquired on Finnigan Mat TSQ 7000 instruments. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were carried out on an Agilent 6540Q-TOF LCMS equipped with an electrospray ionization (ESI) probe operating in positive-ion mode with direct infusion. Dynamic light scattering (DLS) measurements were performed on a NanoBrook 90Plus Zeta (Brookhaven Instruments Corporation, USA) with a 200-mW polarized laser source (λ = 514 nm). Zeta-potential measurements were carried out at 25 °C on a NanoBrook 90Plus Zeta (Brookhaven Instruments Corporation, USA). The UV-Vis absorption spectra were recorded on a Perkin Elmer Lambda 35 UV-Vis Spectrometer. The excitation and emission spectra were measured on a SHIMADZU RF-5301PC Fluorescence Spectrometer. Transmission electron microscope (TEM) were carried out on a HITACHI HT-7700 instrument.

2. Experimental procedure

Synthesis of FITC-labelled glucose oxidase (FITC-GOx). FITC-GOx was prepared according to the reported method.[S1] Briefly, FITC in DMSO (25 μL, 10 mg/mL) was added into GOx aqueous solutions (1 mL, 10 mg/mL containing 100 mM sodium carbonate). After incubation at 4 ^{o}C for 12 h, deionized water dialyzed the reaction mixture overnight in the dark, followed by lyophilization. FITC conjugated to GOx were determined by the extinction coefficients of 44,100 M^{-1} cm^{-1} at 280 nm (GOx), and 81,000 M^{-1} cm^{-1} at 495 nm (FITC).

Fabrication of supramolecular unloaded vesicles (NPs) and GOx-loaded vesicles (GOx@NPs) based on WP6⊃Azo-G complex. Typically, a certain amount of compound **Azo-G** was dissolved in 1 mL DMF to obtain stock solution (1×10^{-2} M), then 50 μL **Azo-G** solution was quickly injected to three-distilled water solution of **WP6** at different molar ratio of **WP6** and **Azo-G** (1 : 0.5, 1 : 1, 1 : 2, 1 : 4, and 1 : 8). The ultimate volume of above mixed solution was 5 mL. Finally, the mixtures were placed on a shaker for 30 minutes and standing overnight to form stable supramolecular unloaded vesicles that were abbreviated as NPs.

For engineering GOx-loaded supramolecular vesicles, different concentrations of GOx (100, 200, 400, 600, 800, 1000 μg/mL) were dissolved in 5 mL PBS solution (pH 7.4, 1 mM) containing **WP6**, followed by rapid adding the DMF stock solution of **Azo-G** (50 μL, 1×10^{-2} M), the mixture was quickly shaken and placed on a shaker for 30 minutes. The final concentrations of **WP6** and **G** were approximately 25 μM and 100 μM, respectively. The prepared GOx-loaded vesicles stood overnight at room temperature, followed by being purified through dialysis (molecular weight cutoff 300 000) in 3 L PBS solution (pH 7.4, 1 mM) for 24 h to remove organic solvent and unloaded GOx. The PBS dialysate was replaced several times. The GOx-loaded vesicles were successfully prepared and denoted as GOx@NPs. In addition, FITC-GOx loaded vesicles were also prepared according to same procedure for quantifying the GOx loading capacity by fluorescence spectroscopy.

The GOx loading content (wt %) and loading efficiency (%) calculated by the following equation:

Loading content (wt %) = ($m_{GOx\text{-}loaded}/m_{GOx\text{-}loaded} + m_{WP6} + m_{Azo\text{-}G}$) × 100

Encapsulation efficiency (%) = ($m_{GOx\text{-}loaded}/m_{GOx}$) × 100

Where $m_{GOx\text{-}loaded}$, m_{GOx}, m_{WP6} and $m_{Azo\text{-}G}$ are mass of GOx encapsulated into the vesicles and mass of GOx, **WP6**, **Azo-G** added, respectively. The mass of GOx was measured through fluorescence spectroscopy at 520 nm and calculated according to a standard calibration curve in the concentrations from 20 to 100 μg/mL in PBS (pH 7.4, 1 mM).

***In vitro* hypoxia-responsive behaviors.** *In vitro* hypoxia condition was generated by bubbling 100% N_2 gas into the reaction solution for 30 minutes. The fluorescence response of NPs (5 μM) to rat liver microsomes was measured at the concentrations of 100 μg/mL in PBS (0.01 M, pH = 7.4) containing 1 mM NADPH. After 1 h incubation at 37 °C under hypoxia environment, the spectra were recorded which were excited at 560 nm and emission at 560 – 800 nm. The slit width was 10 nm for both excitation and emission. Additionally, interferences of various bio-substrates (1 mM of each species) towards NPs (5 μM) was also monitored under hypoxic and normoxic conditions, including NADPH, sodium ascorbate, glutathione (GSH), Cysteine (Cys), homocysteine (Hcy), $MgCl_2$, $FeCl_2$, H_2O_2, and nitroreductase.

Intracelluar imaging of NPs under hypoxia. The hypoxia condition was generated with an AnaeroPackTM (Mitsubishi Gas Chemical Campany, Inc.) and rectangular jar. HL-7702 and HT-29 cells were seeded in confocal dish at the density of 4×10^4 cells per dish and cultured under hypoxia for 24 h, then NPs (10 μM) was added to both types of cells. After incubated for 6 h, cells were washed with PBS for three times, and measured with a confocal microscope.

***In vitro* esterase-responsive drug release behaviors.** Typically, 2 mL of NPs (1×10^{-4} M) was treated with/without porcine liver esterase (4 mg, 30 U/mL) at different

pH environment (pH=5.0 or 7.4) and mixture solution was kept at 37 °C. At timed intervals, 10 μL of mixture solution was taken and added to 1.5 mL acetonitrile to terminate enzyme reaction. The release amount of Cb was determined using HPLC method.

Quantification of H_2O_2 production. The amount of produced H_2O_2 by the GOx@NPs (1×10^{-4} M) was assessed through a colorimetric detection. Briefly, GOx@NPs (1 mL), HRP (150 mU/mL) and TMB (1 mM) were incubated for 30 min at 37 °C, followed by adding glucose (1 mg/mL). UV-vis spectrophotometer was utilized to measure the absorbance at 650 nm at different time points. The concentration of H_2O_2 was calculated according to the standard curve of H_2O_2 concentrations.

Cellular internalization and distribution. HT-29 cells were seeded in confocal dish at a density of 4×10^4 cells and cultured for 24 h before treatment. Next, the original medium was replaced with glucose/serum-free media for incubation 2 h. Then FITC-GOx@NPs (10 μM) was added into cells for treatment 2 h or 4 h. Then, cells were washed with PBS to remove FITC-GOx@NPs completely. Afterwards, LysoTracker Red DND-99 (Thermo Fisher, USA) and Hochest 33342 (Thermo Fisher, USA) were added to stain lysosomes and nuclei, respectively. Finally, the celluar internalization and distribution was observed by fluorescence microscopy (TCS SP8, Leica). The fluorescence characteristics of FITC-GOx@NPs were used to directly monitor without additional dye.

***In vitro* cytotoxicity assay.** HL-7702 normal cells (human liver cell lines) were seeded in 96-well plates at a density of 1×10^4 cells per well (100 μL in DMEM) for 24 h. Afterwards, different amount of **WP6** and **Azo-G** (100 μL) were added into each well to co-culture 24 h. And then, addition of MTT solution (5 mg/mL, 10 μL) into per well to incubate for another 4 h. Finally, 100 μL of DMSO was added to detect the absorbance at 490 nm according to the microplate reader (Thermo Scientific).

Further, intracellular cytotoxicity assays were performed. Briefly, HT-29 cancer cells and HepG2 cancer cells were seeded in 96-well plates at a density of 1×10^4 cells per well for 24 h, respectively. Next, the original medium was replaced with glucose/serum-free media to culture for another 2 h. 100 μL of fresh glucose/serum-free medium containing various amount of Cb, GOx, Cb+GOx, NPs, and GOx@NPs were treated to cells for 2 h. Undelivered sample was gently washed out at once by PBS, and the cells were further cultured with glucose/serum-free media for 30 min. Then, the cells were incubated with glucose-containing media for 12 h. Subsequently, addition of MTT solution (5 mg/mL, 10 μL) into per well to incubate for another 4 h. Finally, 100 μL of DMSO was added to detect the absorbance at 490 nm according to the microplate reader (Thermo Scientific).

Intracellular ROS measurement. The HT-29 cells were seeded with a density of 1×10^5 cells in confocal dish and incubated with RPMI-1640 medium (1 mL) for 24 h. Next, the medium was discarded and replaced with glucose/serum-free RPMI-1640 containing NPs, GOx@NPs and PBS, respectively. After 2 h, ROS probe (DCFH-DA) was used to detect intracellular ROS according to standard method and imaged by CLSM.

Flow cytometric analysis. HT-29 cells were seeded in twelve-well plates at a density of 1×10^5 cells per well in RPMI 1640 medium (1 mL) and cultured for 24 h. Next, the original medium was removed and incubated with glucose/serum-free RPMI-1640 medium. HT-29 cells treated with PBS, Cb (10 μM), GOx (3 μg/mL), Cb+GOx (Cb: 10 μM, GOx: 3 μg/mL) and GOx@NPs (10 μM) in glucose/serum-free media for 2 h, respectively. Undelivered sample was gently washed out at once by PBS, and the cells were further cultured with glucose-containing media for 12 h. Subsequently, the cells were disposed by trypsinization, centrifugation and resuspension in buffer. Finally, the apoptotic and necrotic cells were examined by Annexin V‑FITC apoptosis detection kit according to the manufacturer's protocol.

Flow cytometer (BD FACS Calibur) was utilized to measure the percentage of necrosis and apoptosis.

Intracellular ATP-level measurements. An ATP assay kit measured the ATP levels in cells. HT-29 cells were seeded in a six-well plate at a density of 80 000 cells per well and cultured for 24 h. The cells were incubated with PBS as control and GOx@NPs (10 μM) at free-glucose or glucose (1 mg/mL) conditions for 6 h. Next, the recommended procedures of the kit determined the ATP levels in cells.

DNA damage evaluation by comet assay. The detailed procedure of measurement was referred to the literature.[S2] HT29 cells were seeded in 6-well plates at a density of 1×10^5 cells per well in 2 mL of complete RPMI 1640 medium and incubated overnight at 37 °C with 5% CO_2 for cell attachment onto the substrate. The medium was replaced with fresh RPMI 1640, followed by adding the aqueous solution of GOx@NPs (10 μM) and PBS as control. After incubation for 12h, the cells were washed by ice-cold PBS for three times, detached by trypsin treatment. Comet assaywas conducted according to themanufacturer's protocols. Briefly, HT29 cells were pelleted and resuspended in ice-cold PBS (1×10^5 cells/mL). Aliquote of 10 μL of cell resuspension were mixed with prewarmed (37 °C) 0.7% low melting point agarose (75 μL), followed by loading onto a fully frosted slide that had been precoated with 100 μL of prewarmed (45 °C) 0.5% normal melting point agarose. A coverslip was then applied to the slide and maintained for 10 min at 4 °C. The coverslip was removed and slide was coated with another prewarmed (37 °C) 0.7% low melting point agarose (75 μL). After solidification for 30 min at 4 °C, the slide was immersed in prechilled lysis buffer for 1 h at 4 °C, followed by rinsing in PBS. Then, the slide was incubated in alkaline electrophoresis buffer (1 mM EDTA, 300 mM NaOH) for 30 min for DNA unwinding, followed by electrophoresis at 25 V for 30 min. After electrophoresis, the slide was rinsed twice in Tris–HCl buffer (0.4 mM, pH 7.5). DNA was visualized by propidium iodide (0.5 μg/mL) and observed under fluorescent microscope (Olympus inverted microscope IX-71). The degree of DNA

damage was quantified by the ratio of comet tail (%) which was calculated through Comet Assay Software Project (CASP).

Animals and tumor model. All animal experimental protocols were approved by the Animal Research and Care Committee of Nantong University. To set up the subcutaneous tumor mouse model, male nude mice (5-6 weeks old) were purchased from the Jiangsu KeyGEN BioTECH Co. (Nanjing, China). 1×10^6 HT29 cells were subcutaneously injected into the BALB/c mice. Animals bearing tumor were established (approximately 3 to 4 weeks) before used for fluorescence imaging.

***In vivo* and *ex vivo* fluorescence imaging.** All animal experimental protocols were approved by the Animal Research and Care Committee of Nantong University. HT-29 tumor-bearing mice were used for fluorescence imaging and before imaging, the tumor-bearing mice were injected intravenously with GOx@NPs (200 uM, 200 uL). First, the mice were placed onto the warmed stage inside of an IVIS light-tight chamber and anesthesia was maintained with 2.5 % isoflurane. All the image acquisitions were performed with Caliper IVIS Lumina II in vivo optical imaging system equipped with excitation filter: 550 nm, and emission filter: 630 nm when the mice were anesthetized at 2 h, 4 h, 8 h, 12 h and 24 h after injection. Then, the mice were sacrificed after imaging. The major organs including the heart, lung, liver, kidney, spleen, intestine, and tumor were collected for *ex vivo* imaging with the fluorescence imaging system as described above.

***In vivo* tumor growth inhibition.** All animal experimental protocols were approved by the Animal Research and Care Committee of Nantong University. The HT-29 tumor-bearing male BALB/c nude mice were randomly divided into four groups with 3 mice in each group after the tumor volume reached 50 mm^3 and this day was set as day 0. These mice were administered intravenously with GOx@NPs (200 uM, 200 uL), NPs (200 uM, 200 uL), GOx (800 ug/kg, 200 uL) and saline (200 uL) for one time on the day 0, 3, 6, 9, 12. The tumor volume were monitored for every 3 days by measuring the two axes (mm) of a tumor (L, longest axis; W, shortest

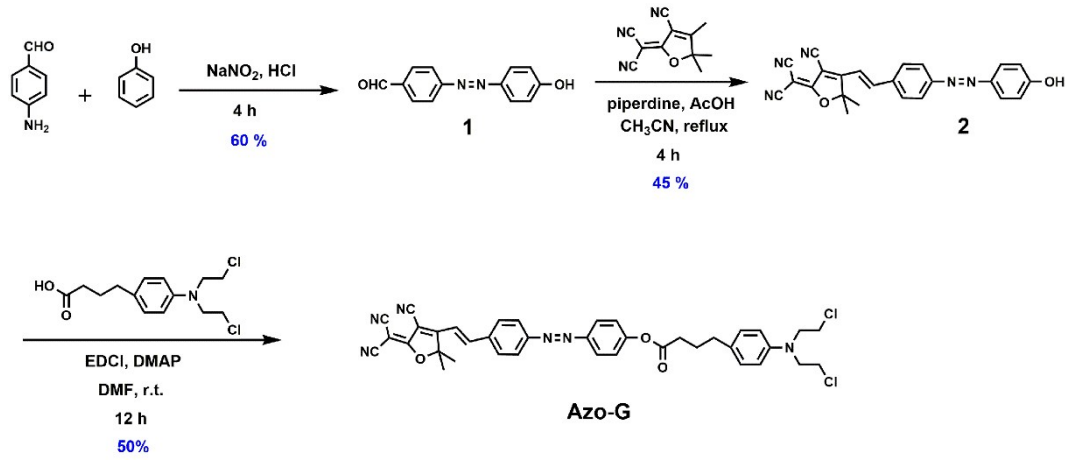
axis) with a vernier caliper and calculated using a formula of ‘tumor volume = ½ ($L \times W^2$)’. The body weight of all animals was also recorded throughout the study. After 15 days post-treatment, the mice were sacrificed and their tumors were dissected out and weighed.

Histological analysis. The representative tumors and major organs of every group were collected at 15 days after treatment and fixed in 4% paraformaldehyde. The paraffin sections were stained with hematoxylin and eosin (H&E). Images were obtained through a 40× magnification (Olympus, Tokyo, Japan) connected to the microscope. The evaluations of histology were performed visually.

Statistical analysis. All results presented are mean ± standard deviation (SD). Statistical analysis was performed using Student's t-test and $P < 0.05$ was considered statistically significant.

3. Synthesis of guest Azo-G

The synthetic procedures for guest Azo-**G** were shown in **Scheme S1**.



Scheme S1. Synthetic route for guest **Azo-G**.

Synthesis of compound **1**

p-aminobenzaldehyde (0.20 g, 1.65 mmol) and concentrated HCl (0.55 ml) were dissolved in water (10 mL) and were kept at 0-5°C for 10 min. $NaNO_2$ (0.11 g, 1.65 mmol) was dissolved in 10 mL water and then added dropwise to the solution of *p*-aminobenzaldehyde. Then the solution of phenol (0.23 g, 2.47 mmol) and NaOH (0.10 g, 2.47 mmol) dissolved in 20 mL water was slowly added to the above solution. The aqueous phase was alkalizated with NaOH solution (1 M) to pH 8. After being stirred for 4 h, the solution was acidized with HCl (1 M) to pH 5-6. The precipitate was collected via filtration and dried to afford a yellow solid (0.22 g, 0.98 mmol, 60 %). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ (ppm): 10.56 (s, 1H), 10.10 (s, 1H), 8.10 (d, J = 8.5 Hz, 2H), 7.98 (d, J = 8.4 Hz, 2H), 7.87 (d, J = 8.9 Hz, 2H), 6.98 (d, J = 8.9 Hz, 2H). LR-ESI-MS: m/z calcd $[M - H]^-$ 225.07, found 225.05.

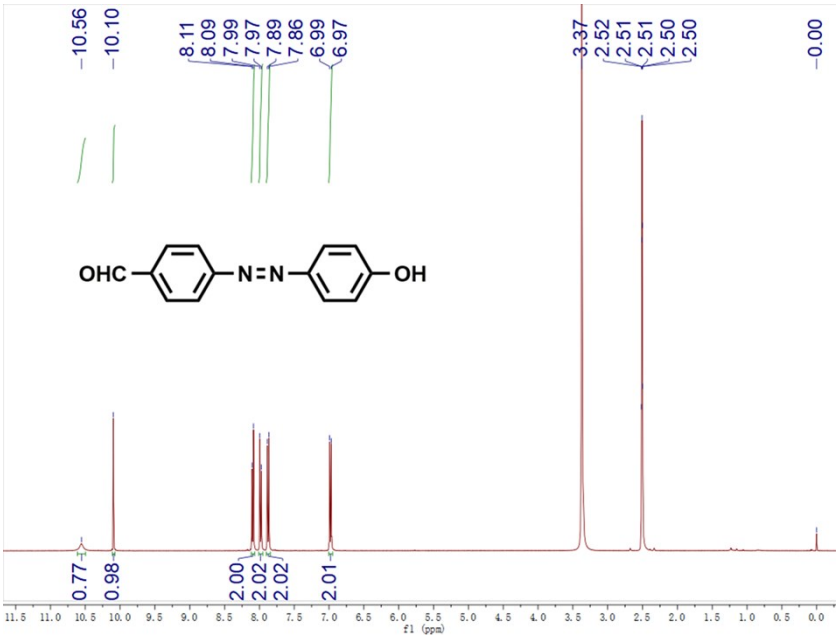


Fig. S1 ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of compound **1**.

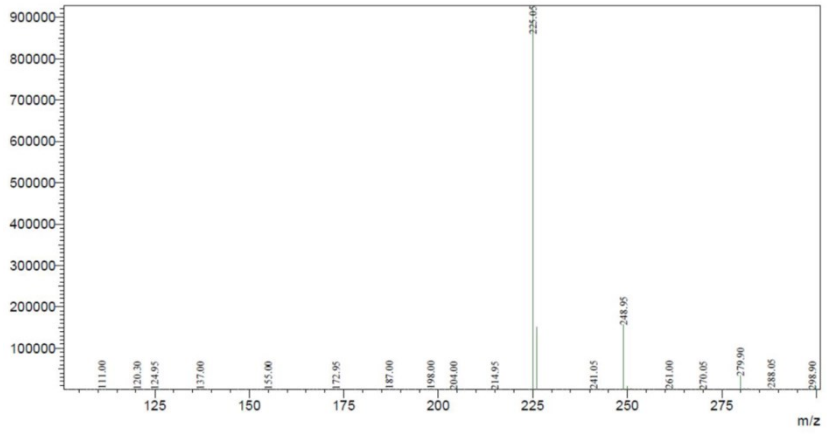


Fig. S2 LR-ESI-MS spectrum of compound **1**.

Synthesis of compound **2**

Compound **1** (0.20 g, 0.88 mmol), piperidine (100 µl), acetic acid (100 µl) and 2-(3-cyano-4,5,5-trimethylfuran-2(5H)-ylidene)malononitrile (0.21 g, 1.06 mmol) were dissolved in 20 mL CH_3CN. The mixture was heated to reflux for 4 h. After cooling down to room temperature, the precipitate was collected via filtration and washed with ethanol several times to afford a purple solid (0.16 g, 0.39 mmol, 45 %). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ (ppm): 10.50 (s, 1H), 8.10 (d, J = 8.6 Hz, 2H), 7.98 (d, J = 16.5 Hz, 1H), 7.92 (d, J = 8.5 Hz, 2H), 7.86 (d, J = 8.8 Hz, 2H), 7.31 (d, J

= 16.5 Hz, 1H), 7.03 – 6.89 (m, 2H), 1.82 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$, 298 K) δ 182.2, 179.9, 166.9, 158.8, 151.2, 150.6, 141.2, 135.8, 130.6, 128.0, 121.5, 121.3, 117.8, 117.0, 116.0, 105.3, 104.7, 59.9, 30.3. LR-ESI-MS: m/z calcd $[M - H]^-$ 406.14, found 406.10.

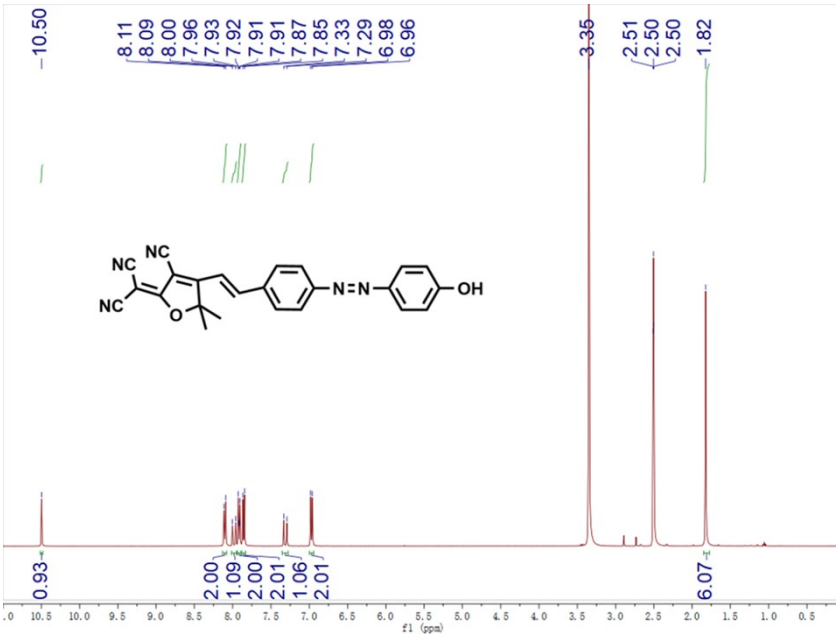


Fig. S3 ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of compound **2**.

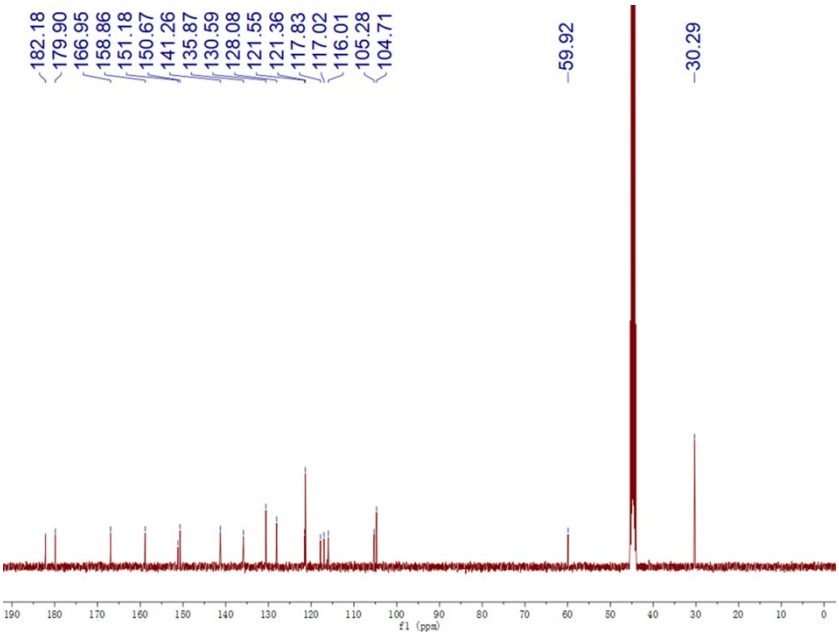


Fig. S4 ^{13}C NMR spectrum (101 MHz, $CDCl_3$, 298K) of compound **2**.

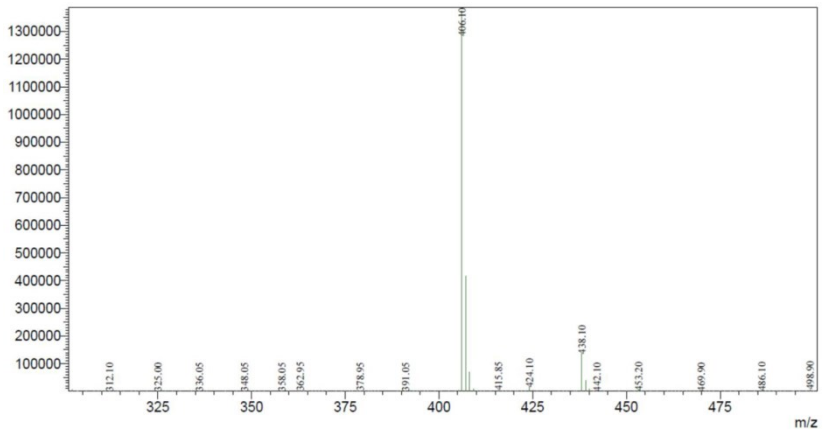


Fig. S5 LR-ESI-MS spectrum of compound **1**.

Synthesis of compound **Azo-G**

A mixture of chlorambucil (0.22 g, 0.73 mmol) and EDCI (0.57 g, 1.47 mmol) in 10 mL of DMF was stirred under argon atomosphere for 0.5 h at room temperature. Then compound **2** (0.20 g, 0.49 mmol) and DMAP (0.03 g, 0.02 mmol) were added into the above solution. The resulting mixture was stirred at room temperature for 12 h. After removal of the solvent under reduced pressure, the crude product was purified by silica gel column chromatography (dichloromethane/ petroleum ether, 1/2 to 2/1) to afford red solid (0.17 g, 0.24 mmol, 50 %). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ (ppm): 8.00 (dd, J = 8.7, 2.8 Hz, 4H), 7.80 (d, J = 8.6 Hz, 2H), 7.70 (d, J = 16.5 Hz, 1H), 7.27 (s, 1H), 7.25 (d, J = 2.0 Hz, 1H), 7.12 (dd, J = 12.5, 3.8 Hz, 3H), 6.66 (d, J = 8.7 Hz, 2H), 3.80 – 3.68 (m, 4H), 3.68 – 3.57 (m, 4H), 2.65 (dt, J = 22.0, 7.4 Hz, 4H), 2.14 – 1.99 (m, 2H), 1.83 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$, 298 K) δ (ppm): 175.0, 173.2, 171.6, 154.4, 153.5, 150.1, 145.9, 144.4, 135.8, 130.1, 129.9, 129.7, 124.5, 123.9, 122.4, 116.0, 112.1, 111.4, 110.7, 110.0, 100.8, 97.7, 53.5, 40.5, 33.9, 33.6, 26.5. HR-ESI-MS: m/z calcd $[M + Na]^+$ 715.1967, found 715.1955.

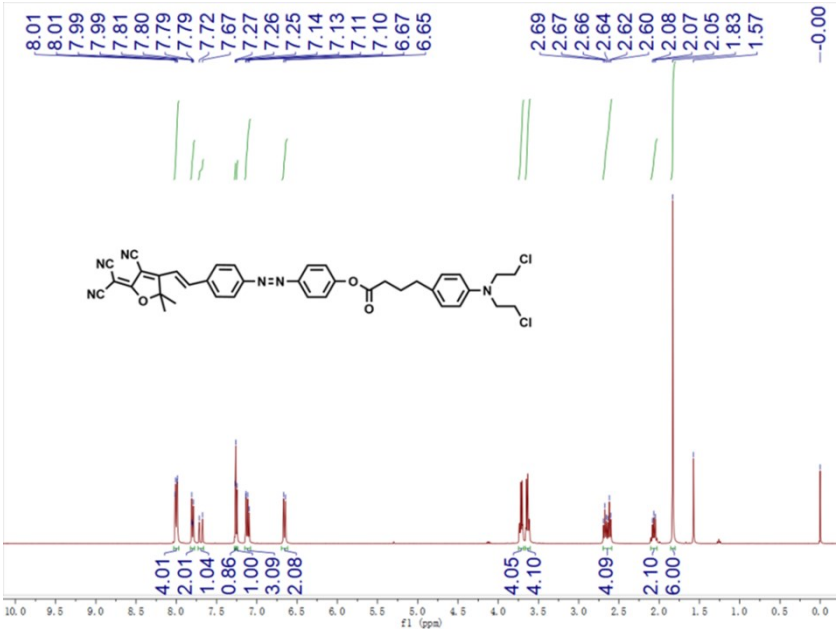


Fig. S6 ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of compound **Azo-G**.

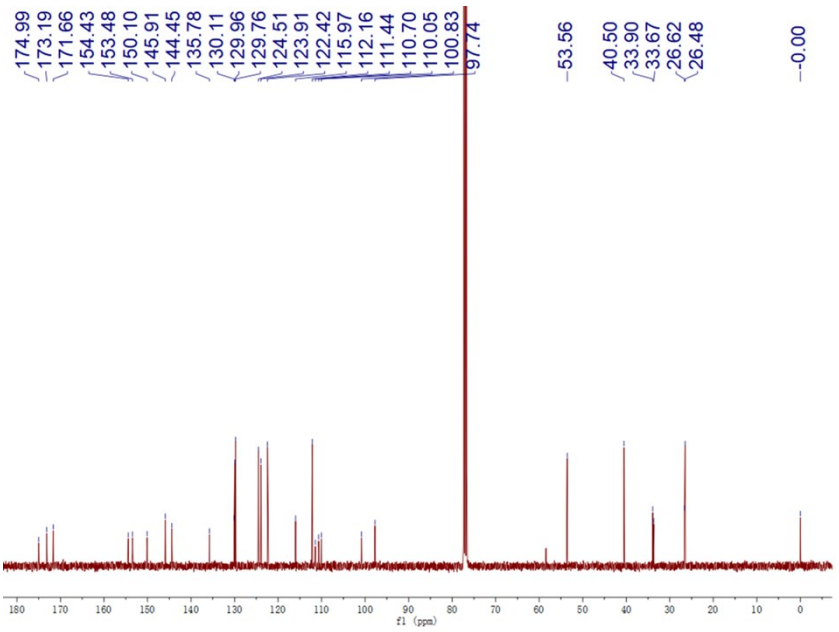


Fig. S7 ^{13}C NMR (101 MHz, $CDCl_3$, 298 K) of compound **Azo-G**.

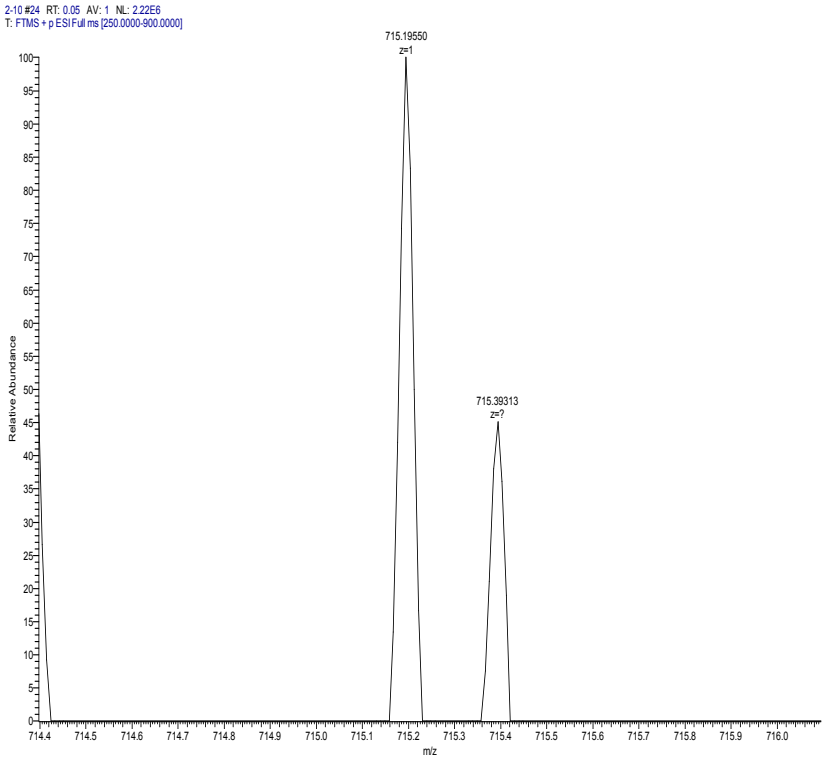


Fig. S8 HR-ESI-MS spectrum of compound **Azo-G**.

4. Stability of the NPs and GOx@NPs

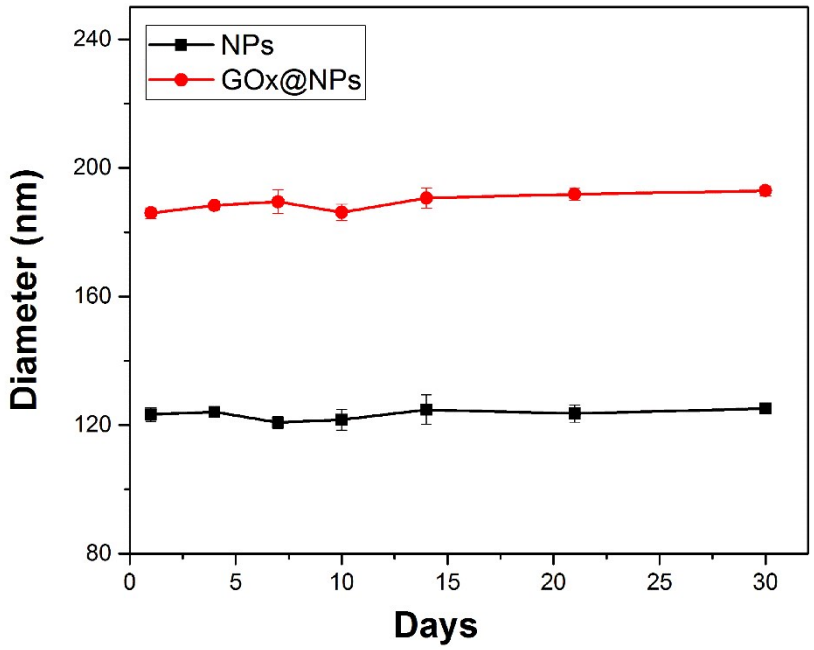


Fig. S9 Time-dependent size changes of **WP6⊃Azo-G** nanoparticles NPs and GOx-loaded nanoparticles GOx@NPs in water.

5. Critical aggregation concentration (CAC) determination of WP6⊃Azo-G

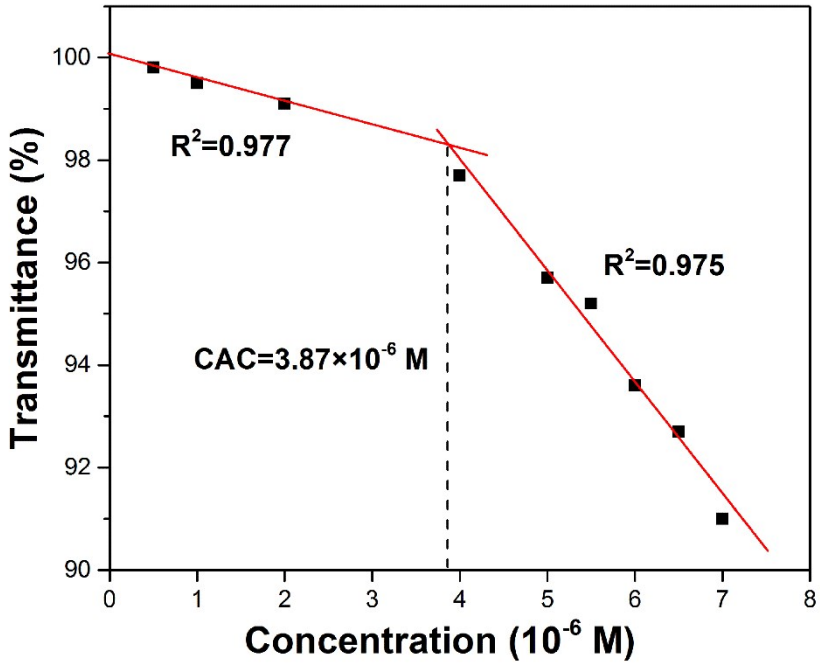


Fig. S10 The concentration-dependent optical transmittance at 560 nm of the **WP6⊃Azo-G** solutions with [**Azo-G**]/[**WP6**] = 4:1.

6. ζ-potentials of the NPs

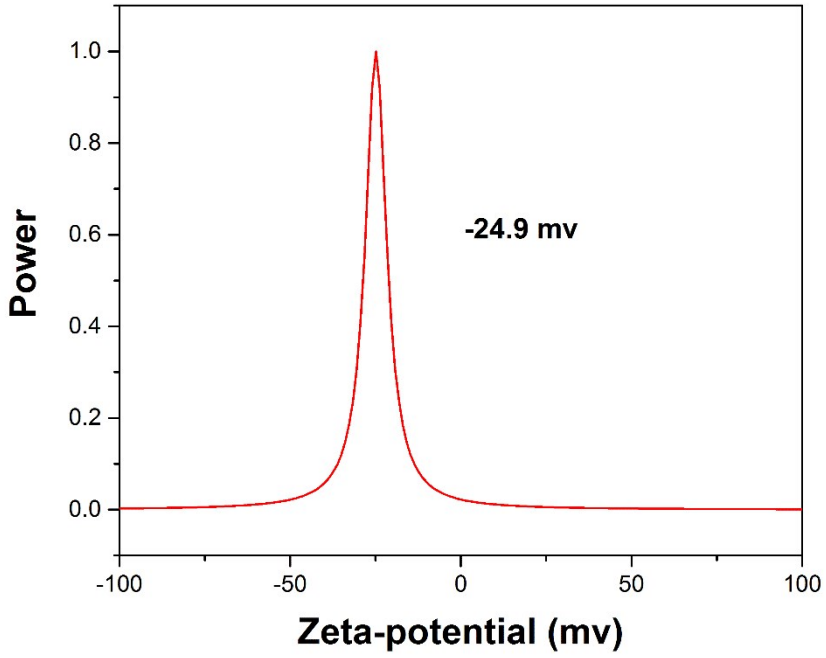


Fig. S11 ζ-potential of the NPs.

7. ζ-potentials of the GOx@NPs

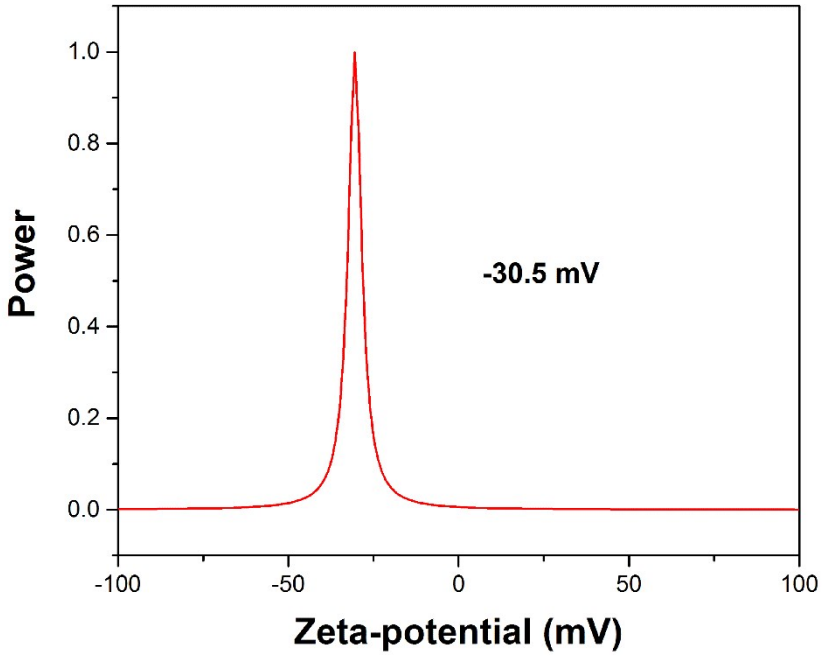


Fig. S12 *ζ*-potential of GOx@NPs.

8. H_2O_2 produnction of the GOx@NPs

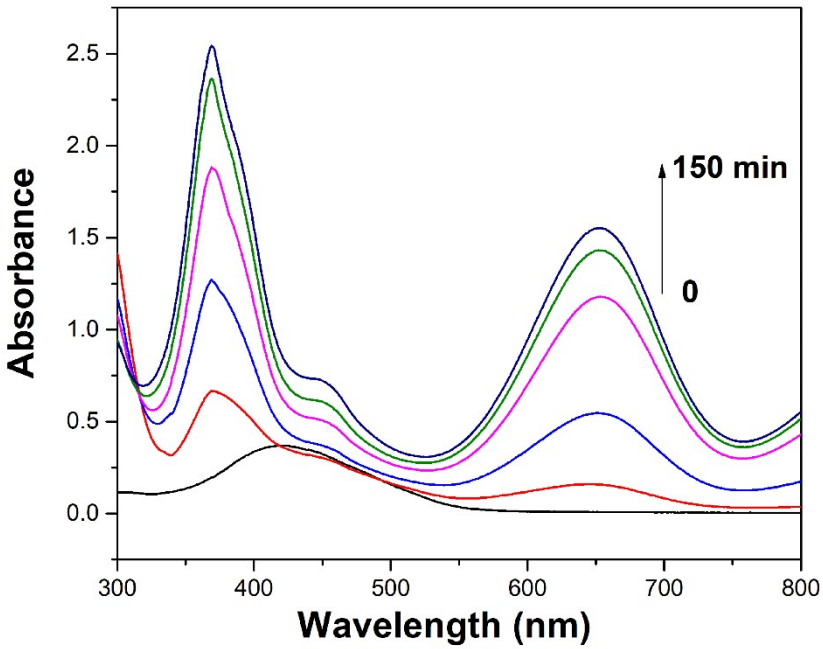


Fig. S13 Change of the absorbance based on oxidative TMB product after incubation of GOx@NPs (1×10^{-4} M) in the presence of 1 mg/mL glucose, 1 mM TMB, and 150 mU/mL HRP.

9. GOx release behavior under different pH values

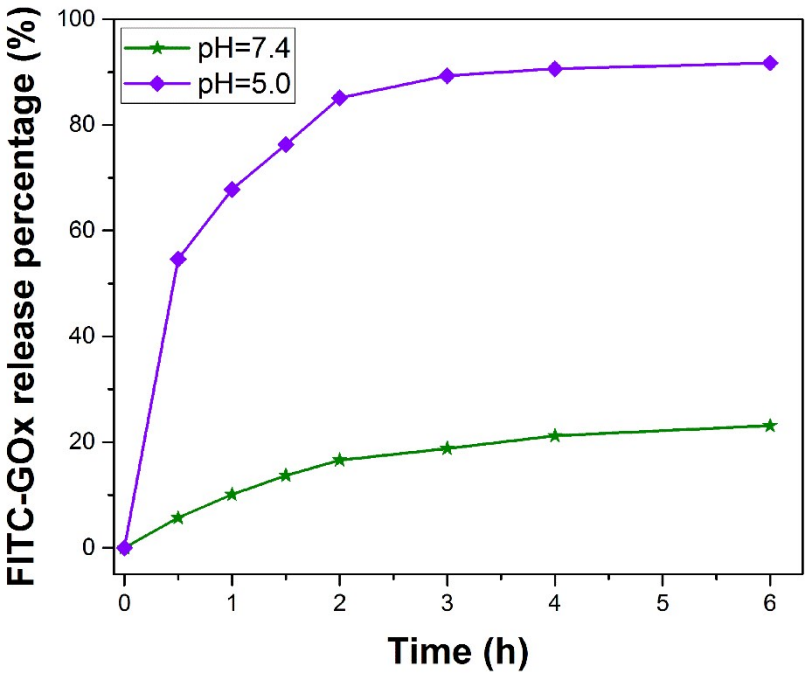


Fig. S14 GOx release profiles from the GOx@NPs under different pH values.

10. In vitro cell assay

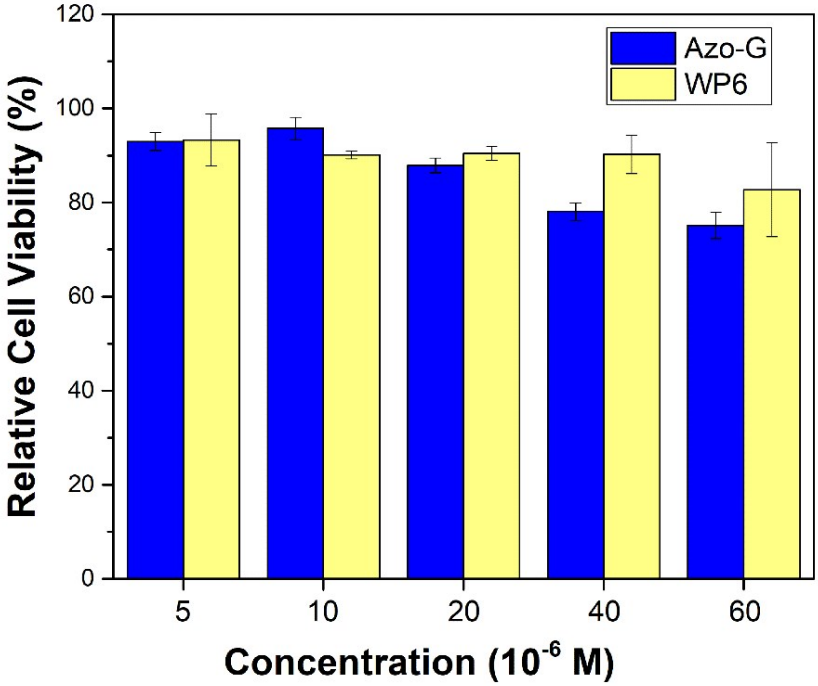


Fig. S15 *In vitro* cytotoxicity of **WP6** and **Azo-G** against HL-7702 cells after incubation for 24 h.

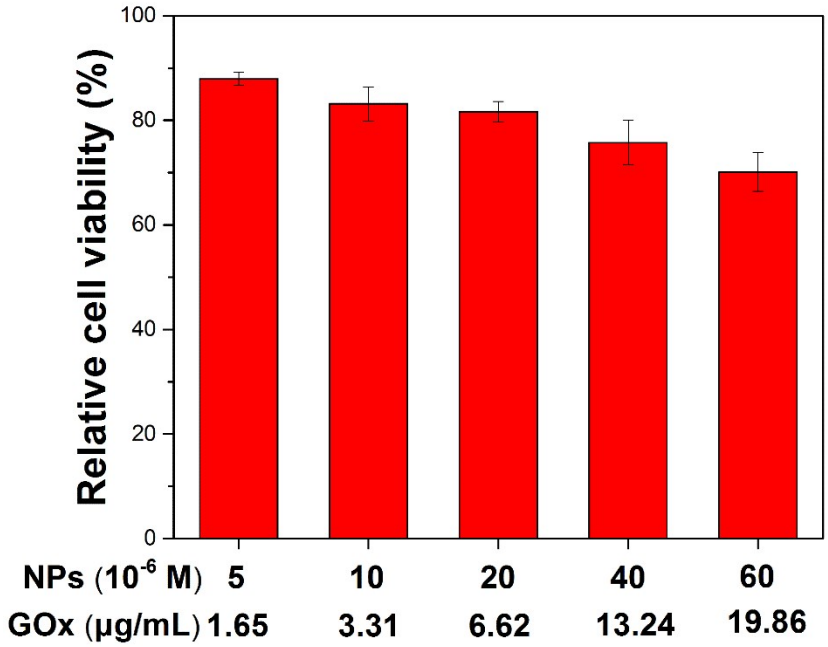


Fig. S16 *In vitro* cytotoxicity of GOx@NPs against HL-7702 cells after incubation for 24 h by intracellular cytotoxicity assays.

11. Blood routine and liver function tests

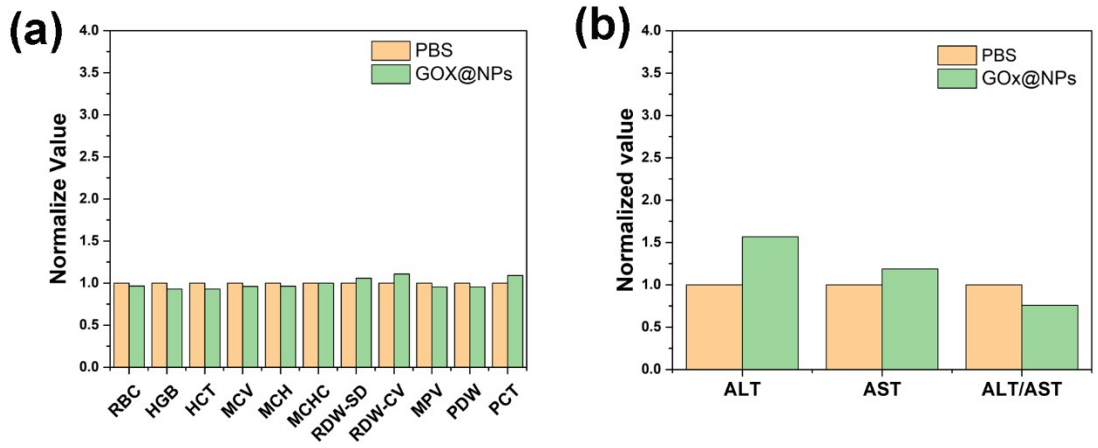


Fig. S17 (a) Blood routine and (b) liver function of analysis with different groups.

12. Reference

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