Electronic Supplementary Information (ESI)

# NO prodrug-conjugated, self-assembled, pH-responsive and galactose receptor targeted nanoparticles for co-delivery of nitric oxide and doxorubicin

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## Materials and methods

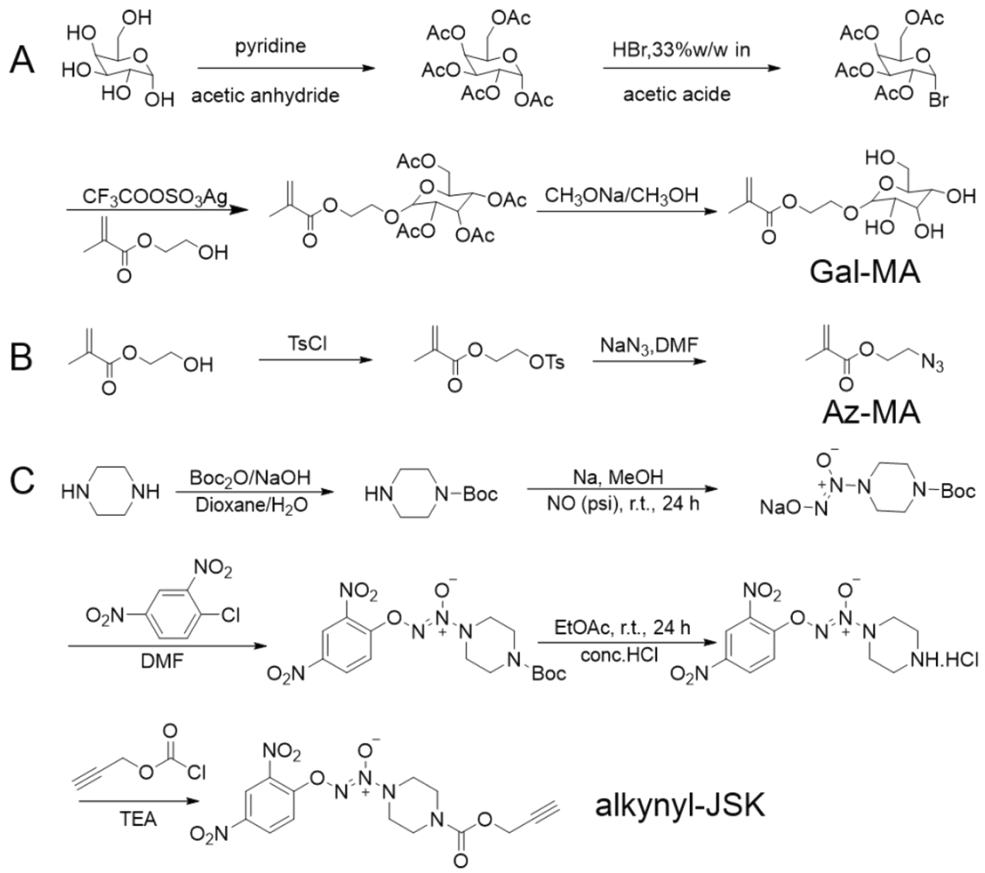
### *Materials*

2-Hydroxyethyl methacrylate(HEMA), D-galactose, 2-(diisopropylamino) ethyl methacrylate (DPA), tosyl chloride(TsCl), acetic anhydride, hydrobromic acid in acetic acid, trifluoroacetic acid silver salt, sodium methanolate, di-tert-butyl dicarbonate and azodiisobutyronitrile (AIBN) were purchased from Alfa Aesar (Lancashire, UK). Benzyl 2-hydroxyethyl carbonotrithioate (BHCT) was a gift received from Dr Pingsheng Huang.
Nitric oxide gas was obtained from sheng tang gas company (Tianjin). Sodium azide, sodium ascorbate, copper(II) sulfate pentahydrate were purchased from tianjin sixth reagent company. Dichloromethane, methanol, tetrahydrofuran (THF), N,N-dimethylformamide(DMF), dimethyl sulfoxide(DMSO) were all analytical grade and used as received from Jiangtian company (Tianjin). Doxorubicin hydrochloride (DOX·HCl) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) and Griess reagent kit were purchased from Beyotime Institute of Biotechnology. All other reagents were commercially available and used as received.

### *Methods*

#### *Synthesis of monomers and NO prodrug (Gal-MA, Az-MA, alkynyl-JSK)*

The synthetic route is depicted in Scheme S1. 2-O-((2', 3', 4', 6'-tetra-O-acetyl)-α-D-galactosyl) hydroxyethyl methacrylate (Gal-MA) and 2-azidoethyl methacrylate (Az-MA) was synthesized according to the standard procedure.[1, 2] The nitric oxide prodrug alkynyl-JSK, $O^2$-(2, 4-Dinitrophenyl) 1-[4-(Propargyloxycarbonyl) piperazin-1-yl] diazen-1ium-1, 2-diolate was synthesized according to the method reported previously.[3, 4]




Scheme S1 Synthesis of (A) Monomer of Gal-MA; (B) Monomer Az-MA; (C) NO prodrug of alkynyl-JSK

***Synthesis of triblock copolymer (p(Gal-b-DPA-b-Az))***

p(Gal-*b*-DPA-*b*-Az) was synthesized via sequential reversible addition−fragmentation chain transfer (RAFT) polymerization of galactosyl methacrylate (Gal-MA, 2× $10^{-2}$ mol, 5.84 g), 2-(diisopropylamino) ethyl methacrylate (DPA-MA, 6 × $10^{-3}$ mol, 1.278 g), and 2-azidoethyl methacrylate (Az-MA, 6 × $10^{-3}$ mol, 0.93 g) in the presence of benzyl 2-hydroxyethyl carbonotrithioate (BHCT, 1 × $10^{-3}$ mol, 0.244 g) as the chain transfer agent and 2, 2-azobisiso butylonitrile (AIBN, 4 × $10^{-4}$ mol, 0.0656 g) as an initiator in DMF.[5] The resulting solution was degassed by three freeze-evacuate-thaw cycles and heated at 60-70 °C for 24 h. The crude product was dialyzed against deionized water for 3 days and finally freezing dried. The chemical structure of p(Gal-b-DPA-b-Az) was analyzed by $^1$HNMR (300 MHz, DMSO) and ATR-FTIR spectroscopy. The composition of the polymers was determined by elemental assay. The molecular weight and PDI of polymers was determined by GPC.

***Conjugation of alkynyl-JSK to AzMA monomer***

155 mg of Az-MA (1 × $10^{-3}$ mol) and 472.8 mg of alkynyl-JSK (1.2 × $10^{-3}$ mol) were diluted in THF (9 mL). 25 mg $CuSO_4$ (2 × $10^{-4}$ mol) and Na-ascorbate 24 mg (4 × $10^{-4}$ mol) were dissolved in $H_2O$ (3 mL). Alkynyl-JSK solution was added to the block copolymer solution under atmosphere protection. Then, the reaction mixture was reacted at room tempreture for 24 h. Meanwhile, DMSO reaction system was also employed and CuBr was used as catalyst in the presence or absence of PMDETA. To this solution, Az-MA, alkynyl-JSK, CuBr, PMDETA was added at the molar ratio 1:1.2:0.1:1. The reaction

mixture was also reacted at room tempreture for 24 h under atmosphere protection. The successful synthesis of AzMA-JSK was achieved by monitoring the new signal at 550.25 Da (AzMA-JSK/$H^+$) and at 572.30 Da (AzMA-JSK/$Na^+$).

***Conjugation of alkynyl-JSK to triblock copolymer***

The NO prodrug conjugated amphiphilic copolymer p(GD-Az-JSK) was synthesized by click chemistry. Briefly, p(Gal-b-DPA-b-Az) ($1 \times 10^{-3}$ mol, 6.3 g) was dissolved in $H_2O$ (15 mL) in the presence of $CuSO_4$ ($5 \times 10^{-4}$ mol, 0.125 g) and sodium ascorbate ($1 \times 10^{-3}$ mol, 0.196 g) under the protection of nitrogen atmosphere. Alkynyl-JSK ($6 \times 10^{-3}$ mol, 6.3 g) dissolved in THF (5 mL) was added to the triblock copolymer solution. The reaction was conducted at 37 ° C for 24 h under atmosphere protection. After the reaction was finished, the products were dialyzed against deionized water for 3 days and finally freezing dried. The entire reaction and dialysis process was performed in dark. The chemical structure of p(GD-Az-JSK) was analyzed by $^1$H NMR (300 MHz, DMSO), ATR-FTIR spectroscopy, UV−vis. pectroscopy, elemental analysis. The molecular weight and PDI of p(GD-Az-JSK) was determined by GPC.

***Preparation and Characterization of p(GD-Az-JSK) NPs and p(GD-Az-JSK)/DOX NPs***

The nanoparticles were prepared by nanoprecipitation methods. Briefly, the conjugated block copolymer with alkynyl-JSK (10 mg) was dissolved in DMSO (500 μL) under moderate stirring at room temperature. This DMSO solution was added dropwise to the water (5 mL) and then stirred for 2 h using a magnetic stirrer. Finally, the reaction was dialyzed (MWCO 1000) for 24 h with changing water every 6 h. The DOX-loaded p(GD-Az-JSK) NPs (p(GD-Az-JSK)/DOX NPs) were prepared using the same method as p(GD-Az-JSK) NPs. First, DOX·HCl was reacted with twice molar amount of NaOH in water. After centrifugation, the precipitation of DOX was obtained and washed with water for three times, which was centrifuged at 4000rpm for 10 min and doxorubicin powder was obtained by freeze-drying. The copolymer p(GD-Az-JSK) (10 mg) and DOX (0.8 mg) were dissolved in DMSO (500 μL) under moderate stirring at room temperature. The mixture was added dropwise in PBS (5 mL) and then stirred for 2 h using a magnetic stirrer. Finally, the resultant solution was dialyzed (MWCO 1000) for 24 h with changing water every 6 h, and p(GD-Az-JSK)/DOX NPs power were obtained by freeze-drying. The stability of p(GD-Az-JSK)/DOX NPs in PBS or DMEM containing 10% serum was studied by DLS within the timeframe of 72 h.

***NO Release in PBS***

The qualitativity of NO release was determined by standard Griess assay, which is widely used for NO determination. The assay was based on the reaction between nitrite, sulfanilamide, and N-1-naphthylethylenediamine dihydrochloride, which can produce a compound with the maximum UV absorption at 540 nm. NO release of p(GD-Az-JSK) NPs with comparison to alkynyl-JSK( 20 μM on alkynyl-JSK basis) was monitored at 37 °C in PBS buffer with different pH in the presence or absence of 10 mM GSH. Griess reagent (50 μl) mixed with the 50 μl sample was incubated for 10 minutes at room temperature. The wavelength scans of UV-vis. spectroscopy was examined. All tests were performed three times.

***NO Release in HepG2 Cells***

The intracellular level of nitric oxide release in HepG2 was also quantified by the standard Griess reagent kit. Briefly, HepG2 cells were seeded in a 96-well plate for 24 h, then cells were treated with p(GD-Az-JSK) NPs or alkynyl-JSK (20 μM on alkynyl-JSK basis) with 150 μL of DMEM medium. At 0, 0.5, 2, 6, 10, 14, 24, 32, 48, 60, 72 h, 50 μL of medium was taken from wells and mixed with 50μL of Griess reagent for 10 min. The nitrite concentration in the sample solution was measured at 540 nm by using a microplate reader, and the total amount of NO release was calculated from a standard curve of nitrite (approximately 1–100 μM) and converted to cumulative NO release.

***Intracellular Release and Distribution of NO by Confocal Microscopy***

NO-sensitive fluorophore 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM DA) was also employed to monitor the intracellular level of nitric oxide in HepG2. HepG2 cells (80000 cells/well) were seeded in 35 $mm^2$ confocal dishes (cover-glass bottom dish) and incubated for 24 h. The medium was then replaced by DMEM medium containing 5μM DAF-FM DA and further incubated at 37 °C for 30 min. Then the cells were subsequently rinsed twice with PBS to remove excess probe. Finally, p(GD-Az-JSK) NPs or alkynyl-JSK (40 μM on alkynyl-JSK basis) was added to the medium. After 4 h incubation, the cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were washed three times with PBS and stained with 4, 6-diamidino-2-phenylindole (DAPI) for 10 min. The cells were further rinsed with PBS, DAPI and NO fluorescence were analyzed using a confocal laser scanning microscopy (Leica TCSNT1, Germany) with the emission at 405 nm and 515 nm.

***Intracellular Release of NO Measured by Flow Cytometry***

The quantitatity of NO fluorescence intensity was determined by flow cytometry assay. Briefly, HepG2 cells were seeded into 6-well plates ($10^5$ cells/well) and cultured at 37 °C for 24 h. The cells were then washed twice with PBS and cultured in DMEM medium containing 5 μM DAF-FM DA at 37 °C for 30 min. p(GD-Az-JSK) NPs or alkynyl-JSK on 40 μM alkynyl-JSK basis were then added to DMEM medium. After incubation at 37 °C for 4 h, the cells were then washed three times with cold PBS, trypsinized and fixed with 4% paraformaldehyde solution. The cells were collected by centrifuging (1500 rpm, 5 min) and analyzed using a four colors BD AccuriC6 (DAPI, 405; NO, 515 nm; DOX, 575 nm).

***pH sensitivity and NO release triggered DOX Release in PBS***

The pH and NO release cooperatively triggered DOX release of p(GD-Az-JSK)/DOX NPs was investigated. The drug release study was carried out in PBS of different pH values of 7.4, 6.45, and 5.0 containing 10 mM GSH. In vitro release studies were conducted by measuring the fluorescence intensities of the micelle solution. To obtain the drug release profile, 5 mL of p(GD-Az-JSK)/DOX NPs (1 mg/mL) were sealed in a dialysis bag (MWCO: 3500) and incubated in 30 mL of PBS at 37 °C under oscillation. 5 mL of release PBS was taken out at scheduled time intervals for testing and replaced with an equal volume of fresh PBS.

***Cellular uptake of p(GD-Az-JSK)/DOX NPs by Confocal Microscopy***

HepG2 cells were seeded in 35 $mm^2$ confocal dishes with $2 \times 10^4$ cells per well in 1 mL of DMEM culture medium and incubated for 24 h. Then, cells were washed twice with

PBS and replaced by fresh DMEM medium containing 5 μM DAF-FM DA and further incubated at 37 °C for 30 min. Then the cells were subsequently rinsed twice with PBS to remove excess probe. To evaluate the hepatic targeting property of p(GD-Az-JSK) NPs, DMEM medium with 20 μM galactose in Gal+p(GD-Az-JSK)/DOX NPs group was further incubated at 37 °C for 30 min. Then fresh medium was added with p(Gal-*b*-DPA-*b*-Az)/DOX NPs, p(GD-Az-JSK) NPs, p(GD-Az-JSK)/DOX NPs, and Gal+p(GD-Az-JSK)/DOX NPs (40 μM DOX, 2.5 μg/mL DOX). After 4 h incubation, cells were washed twice with PBS and fixed with fresh 4% paraformaldehyde for 15 min at room temperature. The intracellular release behaviors of p(GD-Az-JSK)/DOX NPs were further conducted after incubation for 2, 4, 8 and 24 h. The cells were counterstained DAPI for the cell nucleus with an excitation of 405 nm before imaged on fluorescence microscopy (Leica AF 6500), and the fluorescence of NO and DOX were observed under an excitation of 515 and 575 nm respectively.

***Cellular uptake of p(GD-Az-JSK)/DOX NPs by Flow Cytometry***

HepG2 cells were seeded in 6-well plates with $10^5$cells/well in 1 mL of DMEM culture medium and incubated for 24 h. Then, cells were washed twice with PBS and replaced by fresh DMEM medium containing 5 μM DAF-FM DA and further incubated at 37 °C for 30 min. Then the cells were subsequently rinsed twice with PBS to remove excess probe. To evaluate the hepatic targeting property of p(GD-Az-JSK) NPs, DMEM medium with 20 μM galactose in Gal+p(GD-Az-JSK)/DOX NPs group was further incubated at 37 °C for 30 min. Then fresh medium was added with p(Gal-*b*-DPA-*b*-Az)/DOX NPs, p(GD-Az-JSK) NPs, p(GD-Az-JSK)/DOX NPs, and Gal+p(GD-Az-JSK)/DOX NPs (40 μM DOX, 2.5 μg/mL DOX). After 4 h incubation, the cells were then washed three times with cold PBS, trypsinized and fixed with 4% paraformaldehyde solution. The cells were collected by centrifuging (1500 rpm, 5 min) and analyzed using a four colors BD AccuriC6(DAPI, 405; NO, 515 nm; DOX, 575 nm). The cell uptake of p(GD-Az-JSK)/DOX NPs were further conducted after incubation for 2, 4, 8 and 24 h.

***Estimation the Cell Cytotoxicity of p(GD-Az-JSK) NPs and the enhancement of chemo-sensitivity of DOX In Vitro***

The cytotoxicity of p(GD-Az-JSK)/DOX NPs and the enhancement of chemo-sensitivity of DOX were conducted against HepG2 using a CCK-8 assay. HepG2 cells were seeded into 96 well plates at a density of $1*10^4$cells/well and cultured at 37 °C for 24 h. The cells were then washed twice with PBS and replaced by medium containing p(GD-Az-JSK) NPs or alkynyl-JSK at serial concentrations of 16.5, 33, 66, 132, 165, and 264 μM on alkynyl-JSK basis for 24, 48, 72 h respectively. Then cells were washed twice with PBS and the viability of HepG2 cells was determined using CCK-8 assay. Afterward, the absorbance of the solution was measured at 450 nm using a microplate reader. The results were expressed as a percentage of the absorbance of the blank control. All the experiments were performed in triplicate. The enhancement of chemo-sensitivity of DOX were also conducted with p(GD-Az-JSK) NPs, p(Gal-*b*-DPA-*b*-Az) /DOX NPs, p(GD-Az-JSK) /DOX NPs against HepG2 for 24, 48 ,72 h using the same method mentioned above. The apoptosis of HepG2 cells was studied by Annexin V-FITC/PI staining kit according to the manufacture's guidelines.

## *Characterization*

$^{1}$HNMR spectra were measured using a Varian Mercury Vx-300 MHz spectrometer. All chemical shifts were recorded in ppm (δ) relative to tetramethylsilane (TMS) at ambient temperature. ATR-FTIR measurements were performed by a Bio-rad FTS6000 by averaging 80 scans with a resolution of 0.075 $cm^{-1}$. Molecular weight (Mn) and polydispersity (PDI) relative to PS were measured on a gel permeation chromatography(GPC) using Malvern Viscotek GPCmax system, which was performed with a porous styrene divinylbenzene copolymer-based column (Org 300×7.8 mm, CLM3009, T6000M, General Mixed), using polystyrene as the standard material for calibration and DMF as the mobile phase. The UV-vis absorption spectra were recorded on a Cary 300 Scan spectrophotometer (Varian). Elemental composition of the polymers were determined by Vario EL cube elementar (Germany). The particle size and zeta potential of NPs in PBS solution were measured on Zetasizer Malvern 3000HS (UK). Samples were purified from dust using a microfilter (0.45 µm) before analyzing. The morphology was examined by using a Hitachi HT7700 transmission electron microscope (TEM), The acceleration voltage is 80 kV. Fluorescence measurements were performed, using a F97 pro fluorescence spectrometer with an excitation wavelength of DOX at 475 nm. CCK-8 assay was determined at 450 nm by using a microplate reader (MultisKAN MK3, Thermo Cop, USA). Cell uptake experiment was analyzed using a confocal laser scanning microscopy (Leica TCSNT1, Germany) and quantitatived by BD Accuri C6 (BD Biosciences, San Jose, CA).

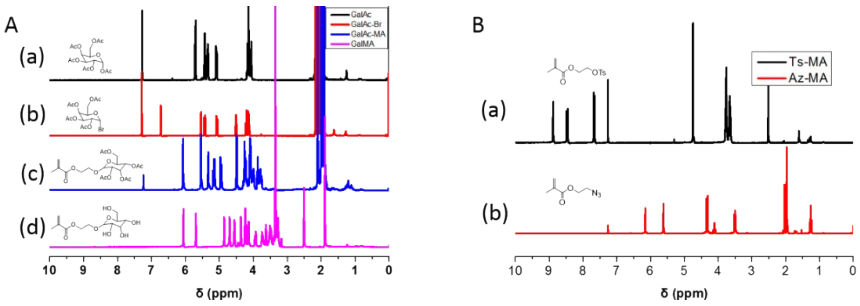



Fig. S1. (A) $^{1}$HNMR spectra of (a) GalAc, (b) GalAc-Br, (c) GalAc-MA, (d) monomer of Gal-MA. (B) $^{1}$HNMR spectra of (a) Ts-MA and (b) monomer of Az-MA.

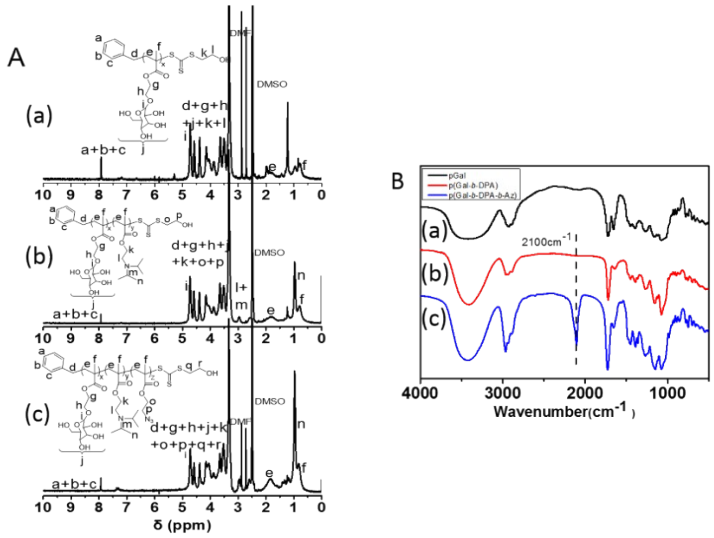



Fig. S2. (A) $^{1}$HNMR and (B) ATR-FTIR spectra of (a) pGal, (b) p(Gal-*b*-DPA), (c) p(Gal-*b*-DPA-*b*-Az).

Table S1. Chemical sructure and composition of copolymers

| Polymers | $M_n^{(a)}$ (g/mol) | $M_n^{(b)}$ (g/mol) | PDI$^{(b)}$ | N$^{(c)}$ |
|---|---|---|---|---|
| pGal | 4610 | 4500 | 1.21 | 15 |
| p(Gal-*b*-DPA) | 5675 | 5600 | 1.23 | 5 |
| p(Gal-*b*-DPA-*b*-Az) | ----- | 6300 | 1.31 | --- |
| p(GD-Az-JSK) | 8420 | 8200 | 1.42 | 5 |

$^{(a)}$Molecular weight determined by $^{1}$HNMR. $^{(b)}$Molecular weight and polydispersity estimated by GPC.

$^{(c)}$Number of block per polymer chain calculated by HNMR.

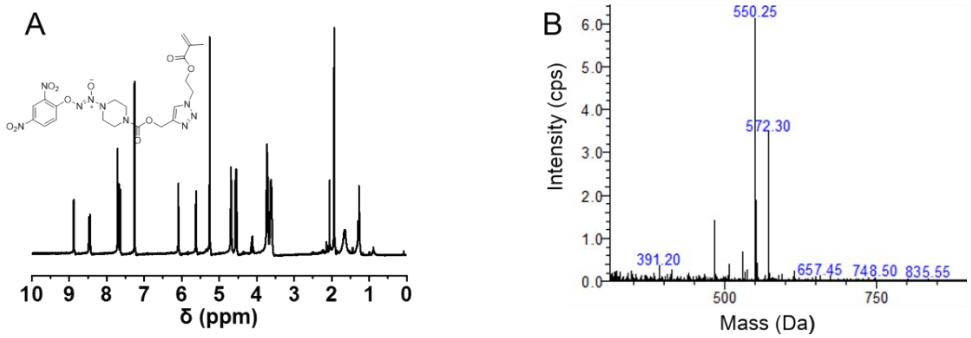



Figure S3. (A) $^{1}$HNMR and (B) ESI spectrum of alkynyl-JSK conjugated to Az-MA monomer (AzMA-JSK).

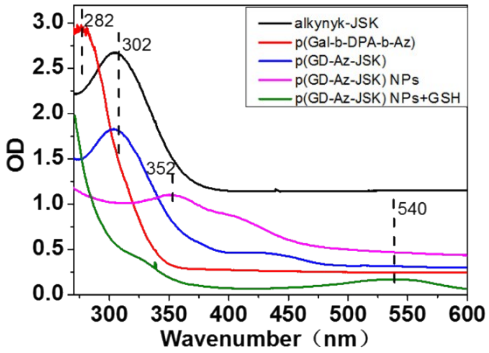



Fig. S4. UV–vis absorption of alkynyl-JSK, p(Gal-*b*-DPA-*b*-Az) copolymer, p(GD-Az-JSK) conjugate, p(GD-Az-JSK) NPs, and p(GD-Az-JSK) NPs in PBS in the presence of 10 mM GSH after 48 h treatment. The presence of an absorption signal at 540 nm (after treatment with Griess reagent) confirmed the NO release in solution.

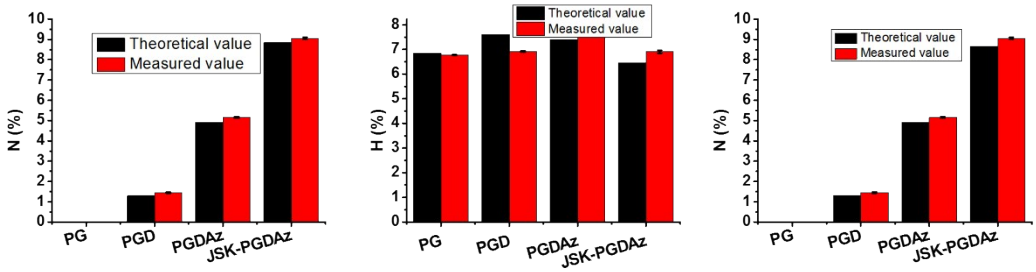



Fig. S5 Elemental analysis of p(GD-Az-JSK): (A) N elements, (B) H elements, (C) C elements.

Table S2. Size (DLS), PDI (DLS), size(TEM), and zeta potential for the polymers in PBS

| Polymers | $SIZE^{DLS}$ (nm) | $PDI^{DLS}$ | $SIZE^{TEM}$ (nm) | ζ ( mv ) |
|---|---|---|---|---|
| pGal | 4 | 0.13 | nd | nd |
| p(Gal-*b*-DPA) | 29 | 0.17 | 25 | -0.374 |
| p(Gal-*b*-DPA-*b*-Az) | 36 | 0.31 | 28 | -0.906 |
| p(GD-Az-JSK) | 45 | 0.38 | 38 | -1.59 |

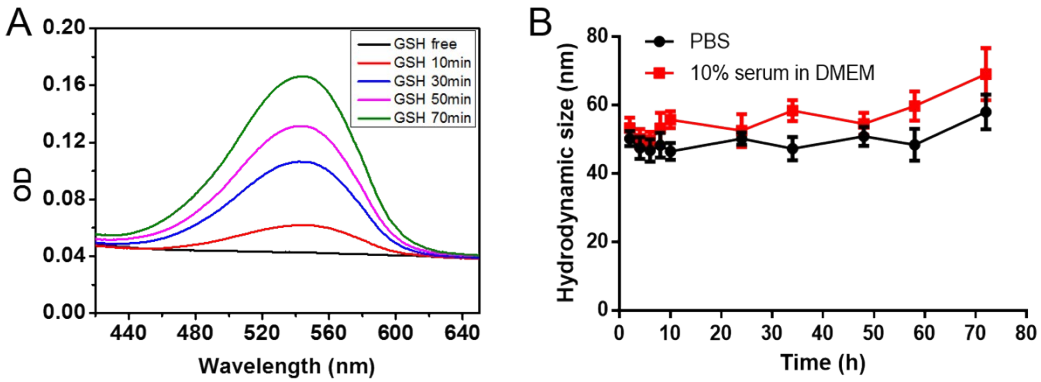



Fig. S6. (A) NO release of p(GD-Az-JSK) NPs in PBS at pH=5.0 values with10 mM GSH at different points (pretreatment with Griess reagent). (B) Hydrodynamic size of p(GD-Az-JSK)/DOX NPs in PBS or 10% blood serum.

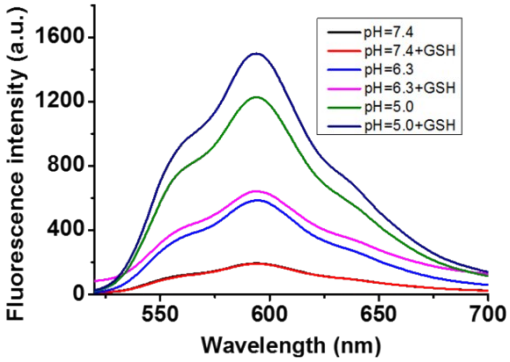



Fig. S7. DOX fluorescence intensity changes of p(GD-Az-JSK)/DOX NPs in PBS at different pH with10 mM GSH.

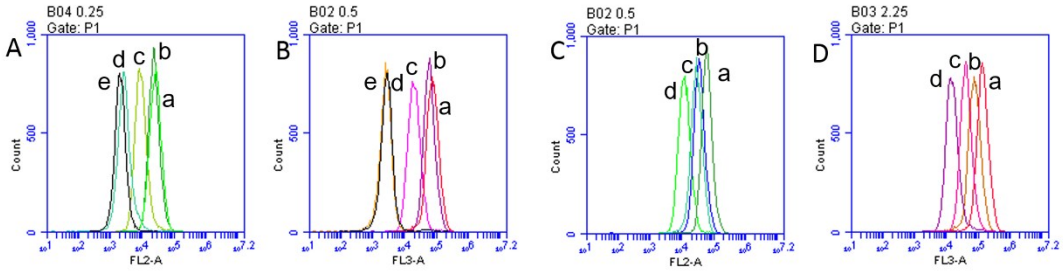



Fig. S8. (A) Flow cytometric quantification of NO or (B) DOX fluorescence intensity after the treatment of various nanoparticles for 4 h; (a) p(Gal-b-DPA-b-Az)/DOX NPs, (b) p(GD-Az-JSK) NPs, (c) Gal+p(GD-Az-JSK)/DOX NPs, (d) p(GD-Az-JSK)/DOX NPs, (e) control. (C) Flow cytometric quantification of NO or (D) DOX fluorescence intensity after p(GD-Az-JSK)/DOX NPs treatment for (a) 24 h, (b) 8 h, (c) 4 h, and (d) 2 h.

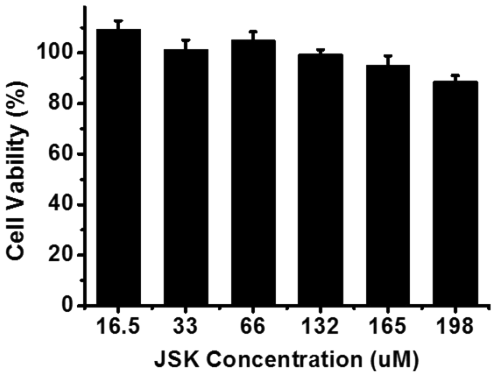



Fig. S9. The cytotoxicity of p(GD-Az-JSK) against 3T3 cell after72 h treatment.

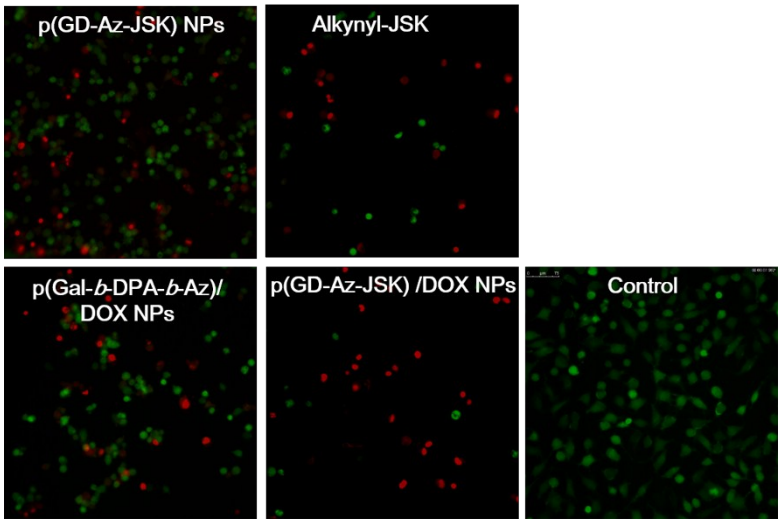



Fig.S10. Fluorescent images of HepG2 cells with live-dead staining after different treatments.

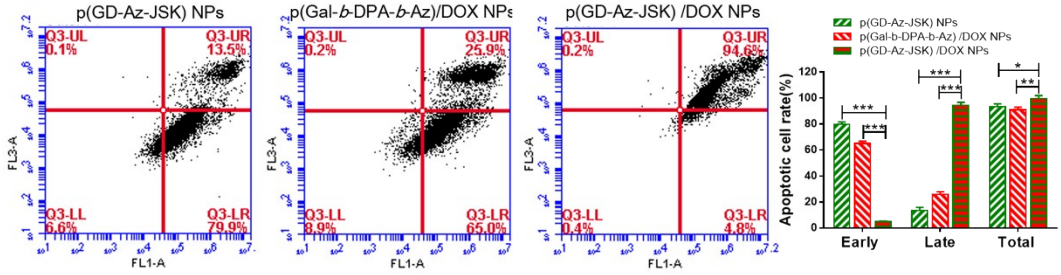



Fig. S11. Annexin V-FITC/PI staining for apoptosis in HepG2 cells treated with p(GD-Az-JSK) NPs, p(Gal-b-DPA-b-Az) /DOX NPs, p(GD-Az-JSK) /DOX NPs (48 μM NO on JSK basis, 3 μM DOX) for 48 h was assessed by flow cytometry analysis. The percentages of early or late apoptosis are presented in the bottom right and top right quadrants, respectively. Columns represent the average proportions of apoptotic cells. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; n=3.

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