Electronic Supplementary Information for

Glucose oxidase triggers gelation of *N*-hydroxyimide-heparin conjugates to form enzyme-responsive hydrogels for cell-specific drug delivery

*Teng Su, Zhou Tang, Hongjian He, Wenjun Li, Xia Wang, Chuanan Liao, Yao Sun, and Qigang Wang**

Contents

1. Materials 2
2. Experimental section 2
2.1 Synthesis of thiol-containing heparin-cysteamine conjugate 2
2.2 Synthesis of *N*-hydroxyimide-heparin conjugate 2
2.3 Synthesis of doxorubicin-bound *N*-hydroxyimide-heparin conjugate 3
2.4 Gel preparation 3
2.5 In vitro drug release 3
2.6 Cytotoxicity assay 4
2.7 Intracellular distribution of the released drug 4
2.8 Statistical analysis 4
3. Characterization 4
3.1 ^{1}H NMR measurement 4
3.2 FTIR spectroscopy 5
3.3 Electron paramagnetic resonance (EPR) measurement 5
3.4 Aqueous GPC measurement 5
3.5 Rheological measurement 5
3.6 UV-vis spectroscopy 5
3.7 Confocal laser scanning microscopy 5
4. Figures 6
5. References 12

1. Materials

N-Hydroxy-5-norbornene-2,3-dicarboxylic acid imide (HONB), glucose oxidase (GOx) from *Aspergillus niger*, heparinase I from *Flavobacterium heparinum*, poly (ethylene glycol) diacrylate (PEGDA, average molecular weight: 700 Da), *N*-(3-dimethylaminopropyl)-*N*'-ethyl carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and cysteamine hydrochloride were obtained from Sigma-Aldrich. Heparin (sodium salt, from porcine intestinal mucosa, average molecular weight: 25 kDa, activity: 150 U mg^{-1}) was purchased from Shanghai Baoman Biotechnology Co., Ltd. (Shanghai, China). Doxorubicin hydrochloride (DOX) was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd. (Zhejiang, China). D-(+)-glucose was purchased from Aladdin. The glucose was dissolved in deionized water to prepare a concentrated stock solution (1.0 M). Prior to use, this stock solution was stored for at least 24 h to reach mutarotational equilibrium. All cell culture media, trypsin-EDTA, fetal bovine serum, penicillin/streptomycin, LysoTracker Green DND-26 were obtained from Life Technologies. All other chemicals were of the highest purity commercially available and were used as received.

2. Experimental section

2.1 Synthesis of thiol-containing heparin-cysteamine conjugate

The heparin-cysteamine conjugate was synthesized by reacting cysteamine with the heparin carboxylic groups based on EDC/NHS activation. The synthesis and purification of heparin-cysteamine conjugate containing ten thiol groups was given as an example. Briefly, heparin (500 mg, 0.02 mmol) was first dissolved in 40 mL of 2-(4-Morpholino) ethane sulfonic acid (MES) buffer (100 mM, pH 6.0) to form a homogeneous solution. Subsequently, 102.7 mg of EDC (at 2.6-fold molar excess to the cysteamine) and 154.1 mg of NHS (at 2.5-fold molar excess to EDC) were added to the solution. After 25 min of carboxylate activation, 23.4 mg of cysteamine hydrochloride (0.206 mmol) was added. The pH of the reaction mixture was adjusted to 7.2 by the addition of 1 M sodium bicarbonate, and the mixture was stirred under Ar atmosphere overnight at room temperature. The reaction solution was dialyzed (MWCO 7000) for 24 h against deionized H_2O. After that, a 10-fold molar excess of DTT was added to the reaction solution and stirred under Ar atmosphere for 5 h at room temperature. Then the reaction solution was dialyzed against 1000 mL of dilute HCl (pH 3.5) containing 0.1 M sodium chloride three times for 1 h, followed by dialysis against 1000 mL of deionized H_2O three times for 1 h. The solution was lyophilized for 48 h to give an off-white solid product in excellent yield (92 %). The purified heparin-cysteamine conjugate was stored at -20 °C prior to use. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 5.34 (s, CH($NHSO_3Na$)*CH*OO), 4.18 (s, CH*CH*(OH)CH), 3.98 (s, CH(CH_2OSO_3Na)*CH*O), 3.67 (s, CH(CH_2OSO_3Na)*CH*OCH(OH)), 3.38 (s, CONH*CH_2*CH_2SH), 2.78 (s, NHCH$_2$*CH_2*SH), 2.01 (s, NHCO*CH_3*).

The number average molecular weight (M_n) of the purified heparin-cysteamine conjugate was approximately 2.58×10^4 g mol^{-1} as determined by GPC (Figure S1). The concentration of thiol groups on the purified product was quantified using Ellman's reagent (ThermoFisher Scientific) following manufacture's protocol (Figure S2).

2.2 Synthesis of *N*-hydroxyimide-heparin conjugate

450 mg of heparin-cysteamine conjugate was dissolved in 18 mL of Tris buffer (100 mM, pH 7.5) to form a homogeneous solution, followed by the activation of thiol groups in the presence of DTT (20 mM). A 5-fold molar excess (to the total thiol groups) of HONB was added to the solution. The pH value was adjusted by 1 M $NH_3 \cdot H_2O$ to 7.5. The reaction mixture was stirred under Ar atmosphere at room temperature for 3.5 h. Then, the mixture was dialyzed (MWCO 7000) for 24 h against 0.1 M sodium chloride, followed by dialysis for 48 h against deionized water. The purified *N*-hydroxyimide-heparin conjugate was obtained after 48 h of lyophilization as an off-white solid and was stored at -20 °C prior to use. Yield: 83 %. ^{1}H NMR (400 MHz,

D_2O) δ (ppm): 10.25 (s, N*OH*), 5.34 (s, CH($NHSO_3Na$)*CH*OO), 4.61 (s, CH(OH)*CH*OO), 4.18 (s, CH*CH*(OH)CH), 3.98 (s, CH(CH_2OSO_3Na)*CH*O), 3.69 (s, CH(CH_2OSO_3Na)*CH*OCH(OH)), 3.38 (s, CONHCH_2CH_2SH), 2.51–2.40 (b, CH_2CH_2SCH), 2.01 (s, NHCOCH_3). IR (KBr): 3416; 2877; 1640; 1544; 1351; 1244; 1097; 951 cm^{-1}.

2.3 Synthesis of doxorubicin-bound *N*-hydroxyimide-heparin conjugate

250 mg of *N*-hydroxyimide-heparin conjugate was dissolved homogeneously in 20 mL of MES buffer (100 mM, pH 6.0). EDC (12.4 mg, 64.7 μmol) and NHS (18.6 mg, 161.6 μmol) were added to the solution, respectively. The reaction mixture was stirred for 25 min at room temperature, followed by adjusting the pH value to 7.2 with 1 M sodium bicarbonate. Then, doxorubicin hydrochloride (12.5 mg, 21.6 μmol), pre-dissolved in 5 mL of MES buffer, was added to the solution. The reaction was performed in the dark overnight at room temperature. The reaction solution was dialyzed (MWCO 7000) against 0.1 M sodium chloride aqueous solution and deionized water in the dark, then lyophilized to give the doxorubicin-bound *N*-hydroxyimide-heparin conjugate as a red solid in excellent yield (93 %). The product was stored at -20 °C prior to use. The content of doxorubicin conjugated to the *N*-hydroxyimide-heparin conjugate was determined to be 4.8 wt % by measuring the absorbance at 485 nm with UV-vis spectrophotometry and correlating with the doxorubicin standard curve.

2.4 Gel preparation

The HepSN gel and the Hep(DOX)SN gel were prepared in a similar procedure. In a typical example of hydrogelation, 50 mg of the *N*-hydroxyimide-heparin conjugate (or the doxorubicin-bound *N*-hydroxyimide-heparin conjugate), 50 mg of PEGDA, and 50 μL of glucose stock solution (1.0 M) were added in deionized water (0.8 mL) under vigorous vortexing to give a homogeneous solution in a sealed glass vial. After the addition of 50 μL of a GOx solution (12 mg mL^{-1}), the vial was allowed to stand at room temperature for about 30 min to give a self-standing hydrogel.

For the preparation of the reference non-responsive PEG-DOX hydrogel, doxorubicin was first conjugated to acrylate-poly (ethylene glycol)-*N*-hydroxysuccinimide (ACLT-PEG-NHS, average molecular weight: 2000 Da, Jenkem) through the reaction between the *N*-hydroxysuccinimide ester at the PEG terminal and the amine in the doxorubicin molecule. Briefly, 25 mg of ACLT-PEG-NHS (12.5 μmol), doxorubicin hydrochloride (10.8 mg, 18.6 μmol), and TEA (3.1 μL, 22.32 μmol) were dissolved in 15 mL of methanol. The reaction solution was stirred at room temperature for 2.5 h, dialyzed (MWCO 1000) against deionized water for 12 h, and then lyophilized to give a red solid product in excellent yield (96 %), denoted as ACLT-PEG-DOX. The PEG-DOX gel was prepared by the photo-polymerization of the monomer precursors as described below. 50 mg of PEGDA, 10.2 mg of ACLT-PEG-DOX, 35 mg of poly (ethylene glycol) methacrylate (PEGMA, average molecular weight: 500 Da, Sigma-Aldrich), and 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, 1.0 wt % relative to the total monomer mass) were dissolved completely in deionized water (0.8 mL). The precursor solution was irradiated by a UV lamp (365 nm, 20 mW cm^{-2}) for 10 min at room temperature. The PEG-DOX gel obtained was washed thoroughly with deionized water to remove any residual chemicals before use.

2.5 In vitro drug release

500 mg of the Hep(DOX)SN gel or PEG-DOX gel that forms randomly shaped particles (approx. 500 μm in diameter) was placed into a dialysis tube (MWCO 10000) containing 2 mL of phosphate buffer (50 mM, pH 6.8) at different concentrations of heparinase I (0, 5 or 10 U mL^{-1}). The dialysis tube was put into a 50 mL-beaker against 20 mL of phosphate buffer (50 mM, pH 6.8) and kept in a horizontal shaker maintained at 37 °C,

170 rpm. At different time intervals, the liquid in the beaker was measured with UV-vis (absorbance at 485 nm) to assess the release of drug from the gel. The release experiments were performed in triplicate.

2.6 Cytotoxicity assay

HeLa cells (a human cervical carcinoma cell line), HepG2 cells (a human hepatoma cell line), and NIH-3T3 cells (a mouse embryonic fibroblast cell line) were obtained from Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences.

HeLa cells were cultured in DMEM/F12; HepG2 and NIH-3T3 cells were cultured in high glucose DMEM. The media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were cultured at 37 °C in a humidified atmosphere with 5 % CO_2, before harvested with 0.25% trypsin-EDTA and rinsed. The cell suspension obtained was used in the following experiments. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based cytotoxicity assay was performed to evaluate the cellular response to different hydrogels according to the previously established method.[1] The cells were seeded on a 24 well plate at a population of 3×10^4 cells per well for 24 h incubation before the tests. The gel particles (approx. 500 μm in diameter) were sterilized in 75 % ethanol for 30 min and irradiated with UV light for another 30 min, followed by rinse with sterilized PBS (10 mM, pH 7.4) thrice and with supplemented cell culture medium thrice. According to the results of in vitro drug release experiments, 60 h was needed for reaching the equilibrium drug release due to the enzyme-induced cleavage of DOX-bound heparin chain in the gel network. Therefore, we investigated the cell viability after 60 h culturing. After incubating the cells with Hep(DOX)SN gel, PEG-DOX gel, HepSN gel, the *N*-hydroxyimide-heparin conjugate, or free DOX at various concentrations for 60 h, 100 μL of MTT solution (5 mg mL^{-1}) was added into each well. Following 4 h incubation at 37 °C (5 % CO_2), the purple formazan product was dissolved in 100 μL of acidic isopropanol containing 10 % SDS and 0.01 M HCl. Resultant dissolvable solution was carefully transferred into a 96 well plate, and its absorbance at 570 nm was recorded with a microplate reader (Infinite M200, Tecan). The data of three independent experiments were presented.

2.7 Intracellular distribution of the released drug

The HeLa or HepG2 cells were cultured in glass bottom dishes (diameter: 20 mm) and incubated with the Hep(DOX)SN gel particles (at DOX concentration of 2 μM) for 20 h at 37 °C (5 % CO_2). The cells were washed with PBS thrice and stained with LysoTracker Green DND-26 (100 nM) for 120 min at 37 °C and 4', 6-diamidino-2-phenylindole (DAPI, 300 nM) for 30 min at room temperature in the dark. The samples were washed with PBS prior to visualization with confocal microscopy.

2.8 Statistical analysis

All experiments were performed independently for at least three times and the results were presented as mean ± SD. Tests for statistical significance were performed using a one-way analysis of variance (ANOVA) followed by Turkey post-hoc test (SPSS 19.0). Single and double asterisks represent $P < 0.05$ and 0.01, respectively. P value < 0.05 was considered statistically significant.

3. Characterization

3.1 ^{1}H NMR measurement

The spectra were recorded using a Bruker ARX-400 (400 MHz) spectrometer at 25 °C. D_2O-substituted sample was used to investigate the polymerization kinetics during the hydrogelation process. The acrylate conversion was calculated by the ratio of acryl protons (CH_2=CH–, δ= 5.6 − 6.5 ppm) to methylene protons of

PEG (–CH_2CH_2O–, 3.65 ppm). In a typical measurement, PEGDA (30 mg), the *N*-hydroxyimide-heparin conjugate (30 mg) and D_2O (0.6 mL) were mixed under vigorous vortexing to give a homogenous solution. After the successive addition of 30 μL of glucose in D_2O solution (1.0 M), and 30 μL of GOx in D_2O solution (12 mg mL^{-1}), the time counting began while the above solution was transferred as quickly as possible to a NMR tube and placed into the probe of the spectrometer. The time-dependent spectra of the sample were recorded *in situ* at pre-determined time intervals using a Bruker Topspin program. In all experiments, the first spectrum was acquired after 5 min reaction.

3.2 FTIR spectroscopy

The infrared spectra were obtained with a Nicolet NEXUS 670 FTIR spectrometer at 25 °C using KBr pellets. Data were collected with a resolution of 4 cm^{-1} and 32 scans.

3.3 Electron paramagnetic resonance (EPR) measurement

The EPR spectra were recorded on a Bruker EMX-8/2.7 Spectrometer operating at 9.873 GHz (microwave power: 20 mW; modulation frequency: 100 kHz; modulation amplitude: 0.5 G; receiver gain: 4×10^5; 25 °C). As for the signals of initiating radical, the mixture of HepSN-GOx-glucose (or HONB-GOx-glucose) ternary initiation system and the spin trap (POBN) in deionized water was rapidly transferred to a standard quartz capillary (1 mm in diameter) and placed into the spectrometer. The spectrum was recorded after 5 min reaction. As for the signals of propagating radical, 5 wt % of the monomer (PEGDA, *N, N*-dimethylacrylamide (DMAA), or PEGMA) was added to the reaction system in the absence of POBN, the spectrum was recorded in the same procedure.

3.4 Aqueous GPC measurement

Gel permeation chromatography (GPC) analysis was performed on a system equipped with an Agilent 1260 Infinity Quaternary pump, a DAWN HELEOS multi-angle laser light scattering detector and an Optilab rEX refractive index detector (Wyatt). Two Agilent PL aquagel-OH columns were used with 0.1 M $NaNO_3$ as eluent at a flow rate of 1.0 mL min^{-1} at 25 °C. The sample was dissolved in deionized water and filtered prior to analysis.

3.5 Rheological measurement

The rheological properties of hydrogels were measured using a Thermo Haake RS6000 rheometer (Thermo Scientific) with parallel plate geometry (diameter: 20 mm). Temperature was controlled to ± 0.1 °C with a thermal controller (SC150-A10) for RS6000. Dynamic time sweep measurements were carried out to measure the storage modulus (G') and loss modulus (G'') as a function of time at a constant frequency of 1 rad s^{-1}. The strain amplitude (γ) was fixed at 1% as it has been found to be well within the linear strain amplitude response regime. The measurements for each sample were performed in triplicate.

3.6 UV-vis spectroscopy

The UV absorbance values of aqueous samples were measured on a UV-2700 spectrophotometer (Shimadzu) at 25 °C.

3.7 Confocal laser scanning microscopy

Confocal microscopic studies were performed on an Olympus FV 1200 confocal microscopy equipped with 405, 488, and 543 nm laser lines using a 40× 1.30 NA oil objective. Images were processed with Imaris (Bitplane) using the maximum intensity projection.

4. Figures

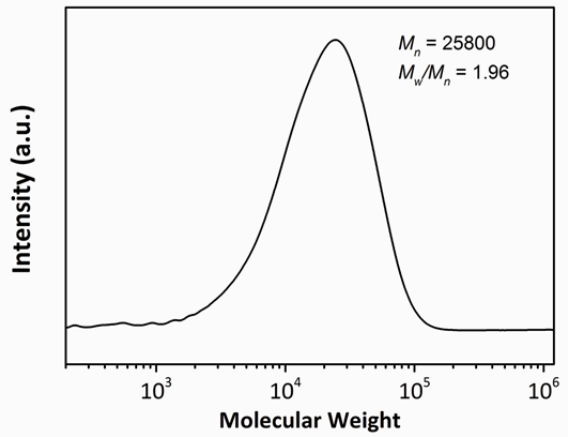

Figure S1 The molecular weight and polydispersity of the purified heparin-cysteamine conjugate containing ten thiol groups.

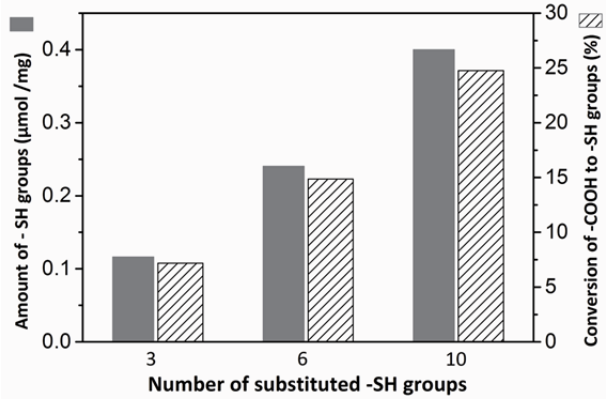

Figure S2 Quantification of the number of thiol groups on the heparin-cysteamine conjugate. Herein, we take the heparin-cysteamine conjugate containing ten thiol groups as an example. The amount of –SH groups on the conjugate was quantified by Ellman's method to be 0.400 ± 0.029 μmol SH/ mg. The M_n of the conjugate was determined by GPC to be 25800 g/mol. Therefore, the number of substituted thiol groups on the heparin-cysteamine conjugate was determined as follows:

$$\text{Number of substituted –SH groups} = \frac{0.400}{1000/25800} = 10.32 \approx 10$$

The original –COOH groups on the backbone of heparin (25 kDa) was estimated to be 41.7, [2] therefore:

$$\text{Conversion of –COOH to –SH groups \%} = \frac{10}{41.7} \times 100\% \approx 24.7\ \%$$

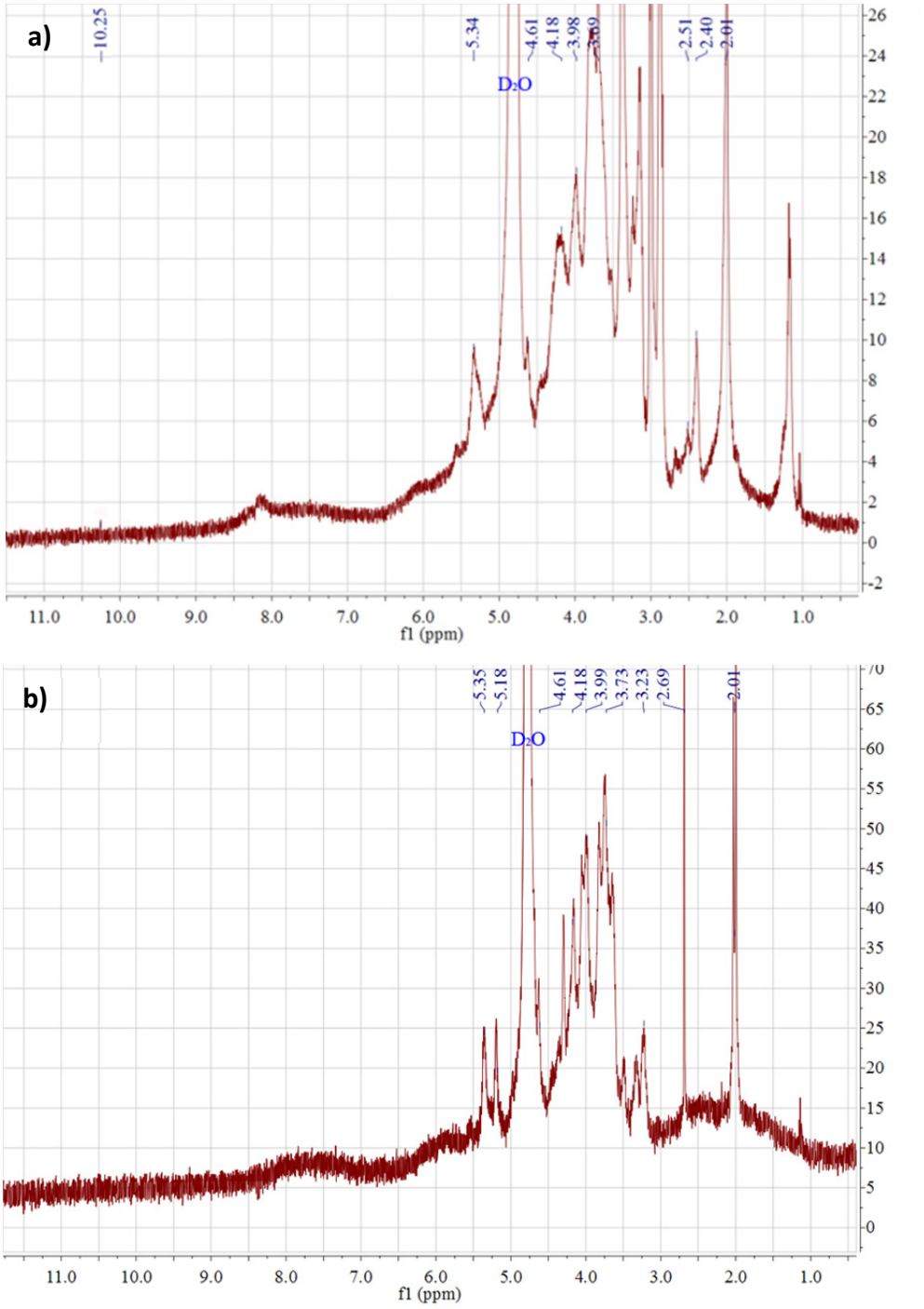

Figure S3 ^{1}H NMR spectra (400 MHz, D_2O) of a) the *N*-hydroxyimide-heparin conjugate, and b) heparin.

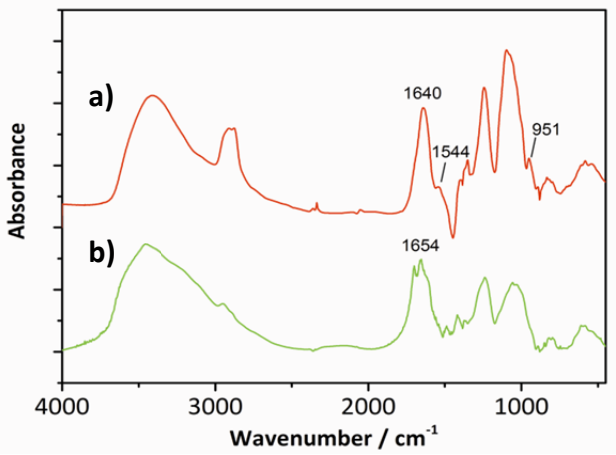

Figure S4 FTIR spectra of a) the *N*-hydroxyimide-heparin conjugate, and b) heparin.

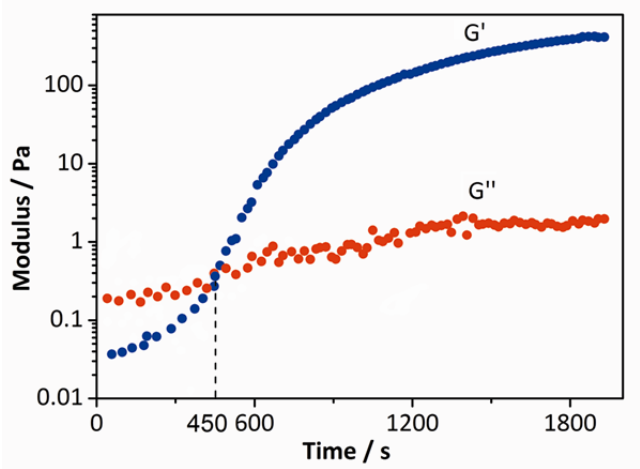

Figure S5 Dynamic time sweep curves of the HepSN hydrogel.

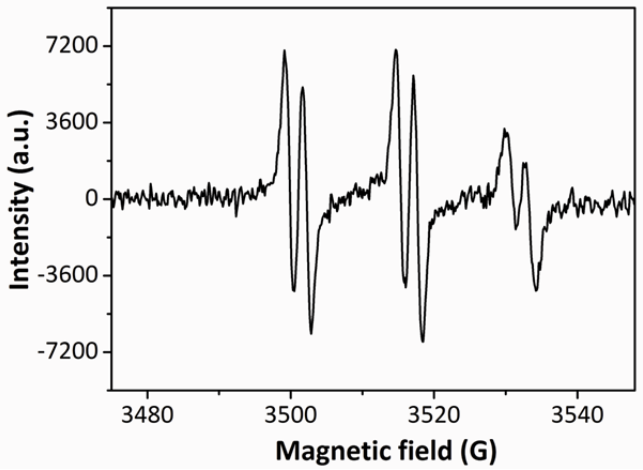

Figure S6 EPR spectrum of the POBN-adducted initiating radical formed in the HepSN-GOx-glucose ternary system aqueous solution after 5 min of reaction.

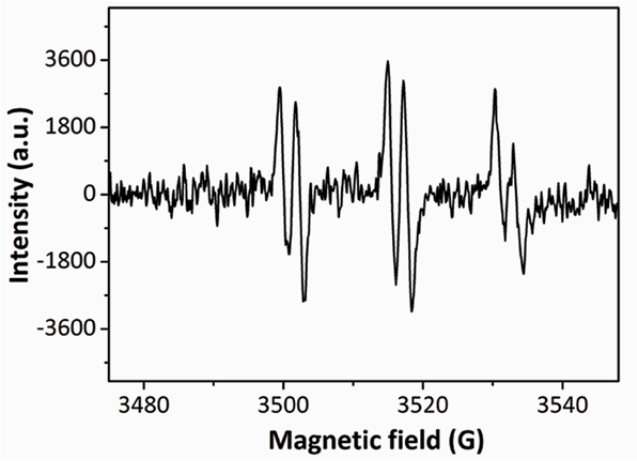

Figure S7 EPR spectrum of the POBN radical adduct formed in the HONB-GOx-glucose aqueous solution after 5 min of reaction.

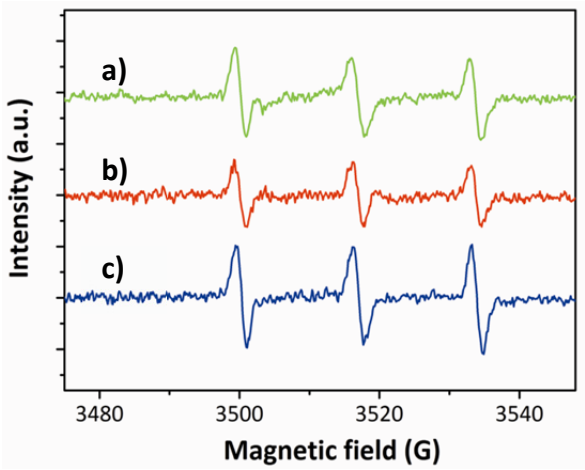

Figure S8 EPR spectra of the HepSN-GOx-glucose aqueous solution with 5 wt % of different vinyl monomers: a) PEGDA; b) PEGMA; c) DMAA in the absence of POBN.

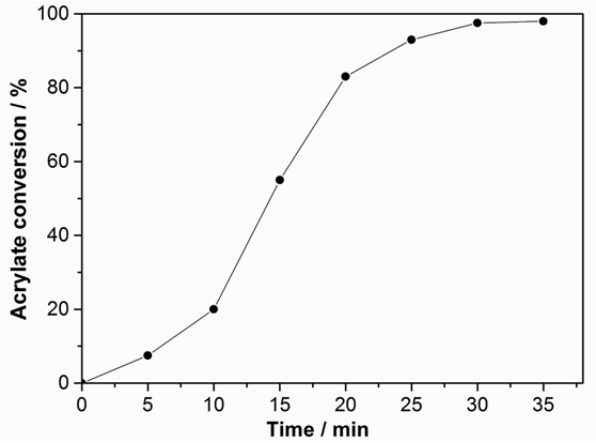

Figure S9 The acrylate conversion in the gel calculated by the peak integral ratio of acryl protons to methylene protons of PEG units.

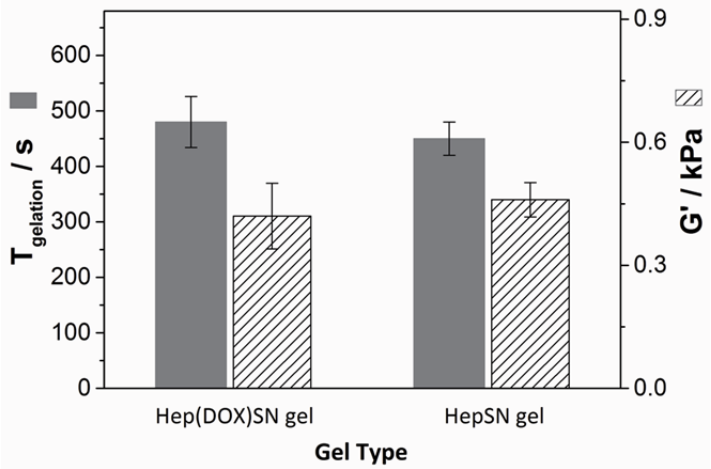

Figure S10 The gelation time and storage modulus of Hep(DOX)SN gel (with 4.8 wt % of DOX conjugation) are similar to those of HepSN gel.

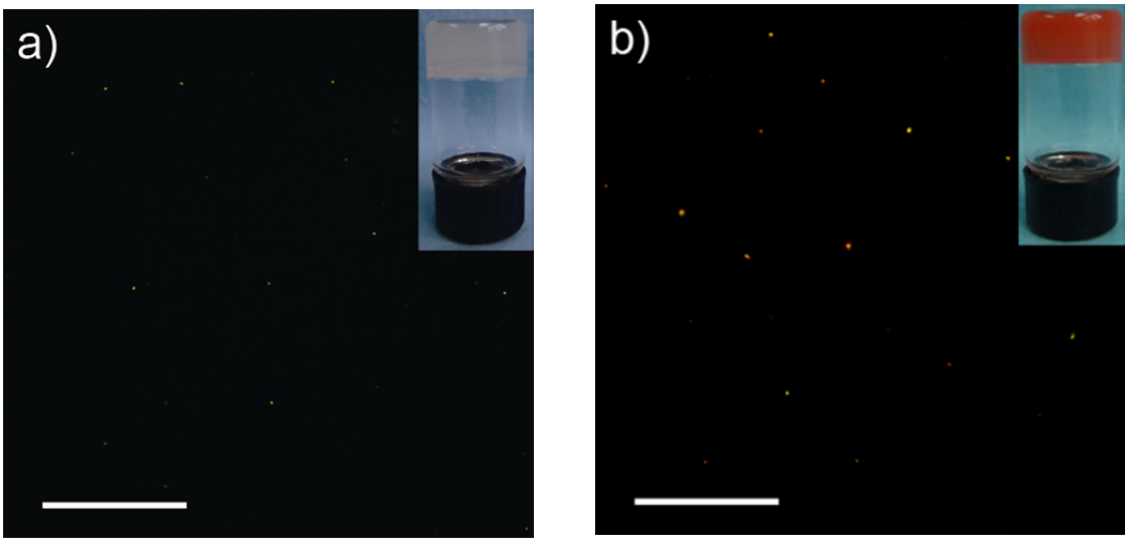

Figure S11 The confocal and optical (inset) images of a) HepSN hydrogel and b) Hep(DOX)SN hydrogel. For visualization by confocal microscopy, the heparin was labeled with fluorescein isothiocyanate (FITC) in the synthesis of the *N*-hydroxyimide-heparin conjugate or the DOX-bound *N*-hydroxyimide-heparin conjugate. The thin layers of hydrogels were prepared in glass bottom dishes (diameter: 20 mm) between a round cover slip (0.13–0.17 mm thick) and the glass bottom. The scale bars are 50 μm.

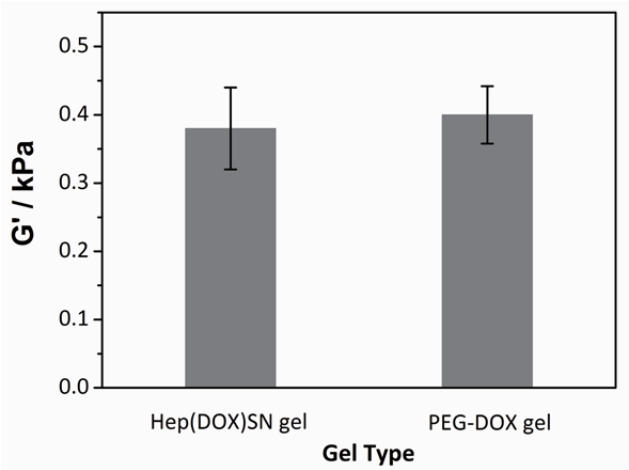

Figure S12 The Hep(DOX)SN gel and PEG-DOX gel with similar storage moduli were used in the drug release experiment.

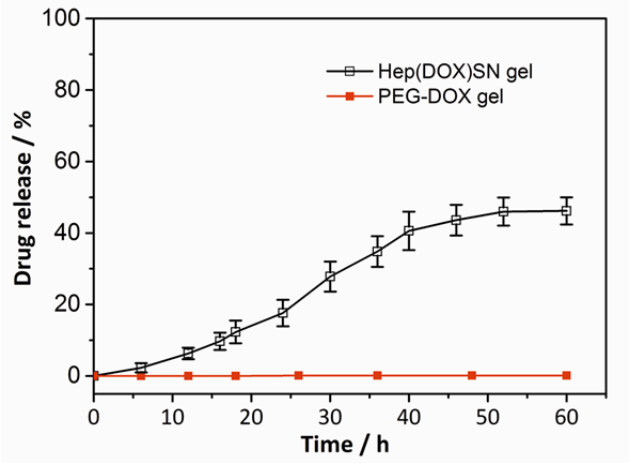

Figure S13 In vitro release profiles of the drug from Hep(DOX)SN gel and PEG-DOX gel in phosphate buffer (pH 6.8) in the presence of heparinase I (5 U mL^{-1}).

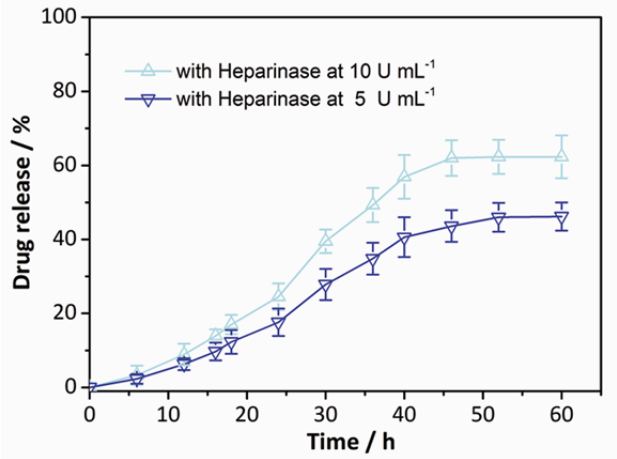

Figure S14 In vitro release profiles of the drug from Hep(DOX)SN gel in phosphate buffer (pH 6.8) at different concentrations of heparinase I (5 or 10 U mL^{-1}).

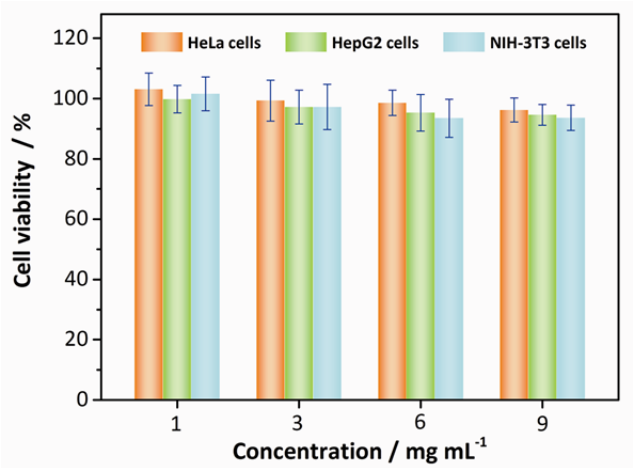

Figure S15 Cytotoxicity of the HepSN hydrogel against different cells. The cell viability was measured by MTT assay after 60 h incubation at 37°C.

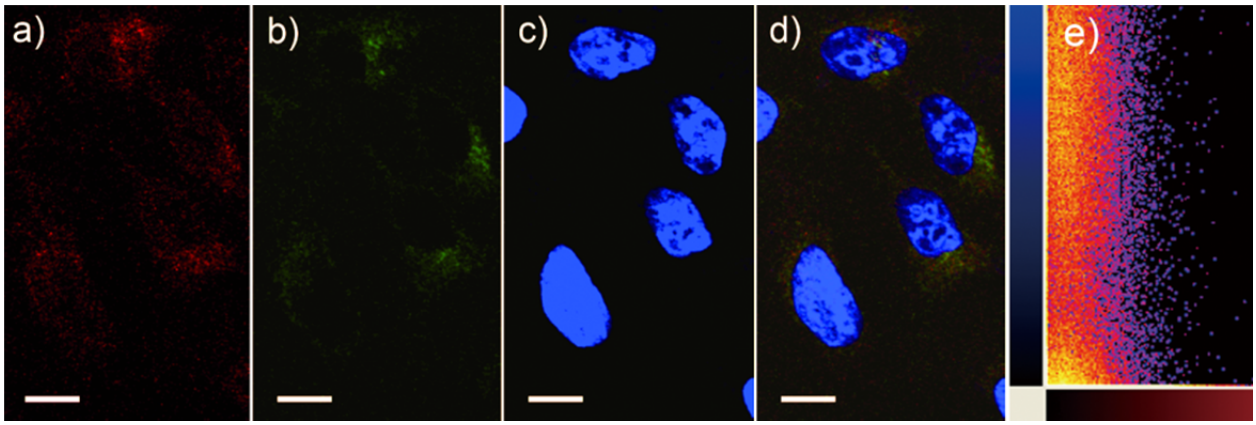

Figure S16 Confocal images (maximum intensity projection) of HepG2 cells incubated with Hep(DOX)SN gel for 20 h. a) released drug, b) lysosomes, c) nucleus, d) overlay, and e) colocalization analysis between DOX and the nucleus calculated from individual fluorescence points. DOX (red), LysoTracker green DND-26 (green), DAPI (blue). The scale bars are 10 μm.

5. References

[1] Z. Fan, B. Liu, J. Wang, S. Zhang, Q. Lin, P. Gong, L. Ma, S. Yang, *Adv. Funct. Mater.* **2014**, doi: 10.1002/adfm.201304202

[2] Z. Zhang, S. A. McCallum, J. Xie, L. Nieto, F. Corzana, J. Jiménez-Barbero, M. Chen, J. Liu, R. J. Linhardt. *J. Am. Chem. Soc.* **2008**, 130, 12998-13007.