Construction of a multi-bioresponsive drug delivery system based on hyaluronic acid and geraniol and evaluation of its antitumor effect on hepatocellular carcinoma

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Experimental details: Materials and methods

1 Materials and Reagents

HA (Mw = 8 kDa) was obtained from Bloomage Freda Biopharm Co., Ltd. (Shandong, China). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), 1-hydroxypyrrolidine-2,5-dione (NHS), adipic dihydrazide(ADH), cystamine dihydrochloride (CYS), geraniol(GER), Succinic anhydride(SA), 4-dimethylaminopyridine(DMAP), 2-[2-[2-chloro-3-[(1,3-dihydro-3,3-dimethyl-1-propyl-2H-indol-2-ylidene)ethylidene]-1-cyclohexen-1-yl]ethenyl]-3,3-dimethyl-1-propylindolium iodide (IR780) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other organic solvents and reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. Fetal bovine serum (FBS) was purchased from GIBCO. Trypsin and high-glucose Dulbecco's modified Eagle's medium (DMEM) were purchased from Beijing solarbio science & technology Co., Ltd. (Beijing, China). All reagents and solvents were used directly.

The experimental Swiss mice (26 ± 2 g) at the age of 6 weeks were purchased from Henan Experimental Animal Center (Zhengzhou, China). The animal procedures were approved by the Animal Experimentation Ethics Committee of Henan University and were performed strictly according to the Guide for the Care and Use of Laboratory Animals and the Regulation of Animal Protection Committee.

2 Synthesis of HSSG and HCCG conjugates.

HA (300 mg, 0.792 mmol), EDC·HCl (182 mg, 0.950 mmol), and NHS (109.3mg,

0.950 mmol) were dissolved in 10 mL of deionized (DI) water at 25 °C under stirring to activate the carboxyl groups of HA. After stirring for 30 min, CYS (535.1mg, 2.376mmol) was introduced into the above activated NHS ester of HA, and then the reaction mixture was stirred for about 24 h.[1] After that, the reaction mixture was poured to a dialysis tube (MWCO 3.5kDa) and dialyzed for 72 h. Finally, the product HA-CYS was collected by lyophilization.

Then, accurately weigh GER (1.54g, 10mmol), SA (1.20g, 12mmol) and DMAP (10mg, catalytic amount) in a 200 ml dry round bottom, and add 50 ml anhydrous dichloromethane. 0.791g pyridine was added under the protection of argon, and the mixture was stirred at room temperature for 24 hours. After the reaction, the mixture was washed with 2 N hydrochloric acid and H_2O in turn, and the organic phase was dried with magnesium sulfate.[2] After removing the solvent, a yellow oily product GER-SA was obtained.

HA-CYS (476mg) was dissolved in 3 mL of formamide under mild heating condition and cooled down to 25 °C. Then, the solution was diluted by 2 mL of DMSO. GER-SA (60 mg, 0.132 mmol), EDC (60.8 mg, 0.396 mmol), and NHS (36.5 mg, 0.396 mmol) were dissolved in 5 mL of DMF at 25 °C. After stirring 30 min, the HA-CYS solution was introduced into the activated GER solution, followed by stirring for 24 h at 25 °C.[3] The final mixture was dialyzed for 72h (MWCO 3.5kDa). HA-SS-GER(HSSG) was collected by lyophilization.

The HA-ADH-GER(HCCG) conjugate used for comparison was synthesized with the same procedure except that CYS was replaced by ADH.

3 Structural Characterization of HSSG and HCCG Conjugates

^{1}H nuclear magnetic resonance (NMR) spectra were recorded using an NMR spectrometer (300 Hz, Bruker, Switzerland). The Fourier transform infrared (FT-IR) spectroscopy spectra were recorded on Bruker IFS-55 (Switzerland). UV−vis spectra were recorded on PerkinElmer Lambda 750 (Norwalk CT).

4 HCCG and HSSG NPs Preparation

The HSSG prodrug was sonicated by a probe ultrasonic sonicator at 0 °C at 150 W and then dispersed in 5 mL of DI water.[4] The ultrasonic dispersion was filtrated through a 0.45μm membrane filter. HCCG NPs were prepared in the same way as the above procedure. The size and surface charge of the particle were analyzed by Malvern Zeta sizer Nano-ZS (Worcestershire, U.K.). The morphology was observed through a JEM 1400 JEOL transmission electron microscope (Tokyo, Japan) at a 200kV acceleration voltage.

The drug-loading content (DLC) was obtained by the equations below

$$\text{DLC (\%)} = \frac{\text{weight of drug in NPs}}{\text{weight of NPs}} \times 100\%$$

The zeta potential, size, and polydispersity index (PDI) in an aqueous solution of these micelles were detected by dynamic light scatting (DLS) on a Malvern Zeta sizer NanoS90 (UK); and the structure and morphology of each micelle were recorded on a transmission electron microscope (TEM, JEOL JEM-1200EX microscope, Japan).

5 Evaluation of nanoparticles stability

To explore the ability of the nanoparticles to maintain stability under storage conditions and in vivo looping environment, nanoparticles were incubated in water,

PBS and PBS containing 10% FBS at 37 ℃.[5] At predetermined time intervals, the size of the nanoparticles was recorded.

6 Multi-bioresponsive drug release profiles of NPs

A dialysis method was used to investigate the multi-bioresponsive drug release behaviors of NPs in different stimulation buffers.[6] Separately, 2mL of HA-functionalized GER-loaded NPs (5mg/ml) were dispersed in various releasing buffers, and the suspension was introduced into dialysis bags with a molecular weight cut-off of 3500 Da. Subsequently, these bags were sealed at both ends and put into centrifuge tubes (50 mL) supplemented with various releasing media (10mL). Meanwhile, SDS was supplemented into the releasing media to maintain the solubility of GER in the aqueous phase, and the final concentration of SDS was set as 0.5% (w/v).[7] Tubes were placed in a thermostat shaker at 150 rpm and 37 °C. At pre-determined time intervals, the releasing media (2 mL) were withdrawn and replenished with the same volume of fresh releasing media. The cumulative amount of GER released was measured at 203 nm using a UV-vis spectrophotometer (Shimadzu RF-5301 PC, Kyoto, Japan). HCCG NPs were used as control.

7 Cell culture

The LX-2 human hepatic stellate cell line and human hepatocellular carcinoma cell lines HepG2 and Huh7 were purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China). All three cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.[8]

8 Cellular Uptake Assay

Fluorescent microscope and flow cytometry could be used to study cellular uptake by a qualitative and quantitative way in respective.[9] Preparation of IR 780-labeled hyaluronic acid nanoparticles: Firstly, carboxylated IR780 was prepared by IR 780 and 6- aminocaproic acid. Next, prepare the HSSG according to the procedures in sections 2.2. Finally, the carboxylated IR780 and the aqueous dispersion of hyaluronic acid nanoparticles HSSG with free amino groups were amidated to obtain IR780 labeled hyaluronic acid nanoparticles (IR780-HSSG). Accurately weigh 10 mg of IR780, dissolve it in 5 ml of methanol, add 8 mg of DCC and 5 mg of DMAP, and stir at room temperature for 1 hour to activate the carboxyl. After the reaction, it was purified by recrystallization. A proper amount was added into the aqueous dispersion of HSSG, and the reaction was performed under magnetic stirring for 24 hours, followed by dialysis (MWCO 3.5kDa) for 48 hours to remove the unreacted IR780 and impurities, and freeze-drying to obtain IR 780-labeled hyaluronic acid nanoparticles IR780-HSSG. The above reaction was carried out in a dark environment.

For fluorescent microscope experiments, the cells were treated with IR780-HSSG (containing 1 μg/mL IR780) for 1, 3, 6, 12, and 24 h at 37 °C. Then, the cells were washed twice with PBS, fixed with 4% paraformaldehyde solution for 20 min, and stained with DAPI for 15 min. Fluorescent images were obtained using a fluorescent inverted microscope (Olympus BX43 CKX31). The excitation wavelength of IR780 was 633 nm and the emission spectrum recorded was between 700 and 800 nm.[10]

As for flow cytometry experimentation, the cells were treated in the same way as fluorescent microscope experiments. Afterward, the cells were washed three times with PBS and resuspended in 500μL PBS cellular uptake efficiency was detected by flow

cytometer (Cyto FLEX, Beckman).

9 Endocytic pathway assay

A total of 3×10^5 HepG2 and Huh7 cells/well were cultured in 6 well plates and incubate for 24 h. The old medium was discarded, and the prepared medium of 10.0μg/mL wortmannin, 50.0μg/mL genistein, 10.0μg/mL CPM, and 10mg/mL HA were added into the plate wells, respectively.[11] After pretreatment in a cell incubator for 1h, the IR780-HSSG NPs solution (containing 1g/mL IR780) was added. Then continuous culture for 3 h, the cells were treated and fluorescence intensity was detected by FCM.

10 In vitro cytotoxicity assay

The effects of hyaluronic acid group (HA), free geraniol group (GER), non-redox geraniol group (HCCG) and redox-sensitive geraniol group (HSSG) on the survival rates of human liver cancer cell lines HepG2, Huh7 and human hepatic stellate cell line LX-2 were evaluated by MTT assay.

Cells in a logarithmic growth phase were inoculated into 96-well plates. After 24 h of cell culture (37 °C, 5% CO2), 100μL drugs of different concentrations were added to the 96-well plate. Three duplicate holes were set for each concentration, and the control group was set. After further cultured for 24, 48 and 72 h, the supernatant was poured out and 20μL MTT solution with a concentration of 0.5 mg/mL was added. After further cultured for 4 h, the supernatant was discarded and 100μL of DMSO was added. The absorbance of each group at 490 nm was measured on a microplate reader (Multiscan, Thermo, USA). The percentage viability of treated cells was calculated and

compared with that of the untreated control cells. Cell viability (%) = OD of drug-treated cells/OD of the culture medium-treated cells × 100%.[12]

11 Dead/Live Staining

HepG2 and Huh7 cells were seeded in cell culture dishes and then incubated with HA, GER, HCCG and HSSG NPs (80μM and 100μM, separately) for 24 h. Then, the treated cells were stained with Calcein-AM (green, live-cell) for 30 min and PI (red, dead cell) for 2-5 min. Finally, the cells were imaged by fluorescent inverted microscope (Olympus BX43 CKX31).

12 Cell proliferation assay

The 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay was performed using the Cell-Light EdU Apollo 567 In Vitro Imaging Kit (RiboBio, Guangzhou, Guangdong, China) following the manufacturer's instructions. HepG2 and Huh7 cells were introduced into a 96-well plate with 2×10^4 cells/well. After 24 hours of drug treatment, they were incubated with 10μM EdU for 2 hours, and then cultured for 24 hours. Then, cells were fixed in 4% paraformaldehyde for 30 minutes, and then treated with 0.5% Triton X-100 solution for 15 minutes at room temperature. Next, 200μL Apollo solution was added to each well and incubated in the dark for 30 minutes. Fluorescent images were acquired using a fluorescent microscope (Olympus BX43 CKX31). Cell proliferation rate (%) = (number of EdU- positive cells)/ (total number of cells) ×100%.

13 TUNEL assay

According to the manufacturer's protocol, the in situ cell death detection kit (Beyotime biotechnology, Shanghai, China) was used for terminal deoxynucleotidyl

transferase-mediated nick end labeling (TUNEL). HepG2 and Huh7 cells were introduced into 96-well plates at a rate of 2×10^4 cells/well, and treated with drugs for 24 hours. After washing by PBS, cells were fixed in 4% paraformaldehyde for 30 minutes, and then treated with 1% Triton X-100 solution at room temperature for 10 minutes. Then, 200μL TUNEL solution was added into each well and incubated at 37℃ for 80 minutes under dark and stained with fluorescent dyes. 4′, 6-diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei at room temperature for 5min under dark. Fluorescent images were acquired using a fluorescent microscope (Olympus BX43 CKX31). TUNEL positive cells were counted using Image J software.

14 In vitro cell apoptosis and cell cycle assays

A total of 3×10^5 HepG2 and Huh7 cells/well were cultured in 6 well plates and incubate for 24 h. Then, the medium was replaced with fresh medium, and the cells were further treated with PBS, HA, GER, HCCG and HSSG respectively for 24 h. Apoptotic cells and cell cycles were detected by staining with an YF®488-Annexin V and PI Apoptosis Detection Kit (US Everbright® Inc, Suzhou, China) or cell cycle kit (Everbright Inc., California, USA), analyzed using software FlowJo. Each assay was repeated in triplicate.

15 JC-1 Staining

Like Calcein-AM/PI staining, after various treatments, the cells were stained with a JC-1 according to the kit. Afterward, we used the (Olympus BX43 CKX31) to capture the fluorescence images.

16 Western blotting

Total protein was extracted from cells. Western blotting was performed to determine the expression levels of target proteins. The primary antibodies, including Anti-Bax, anti-Bad, anti-Bcl-xl, anti-Bcl-2, anti-cleaved caspase-3, anti-cleaved caspase-9, anti-cleaved PARP and anti-GAPDH antibodies were purchased from ProteinTech (Chicago, IL, USA). The horseradish peroxidase-conjugated secondary antibody was purchased from CST. The Proteins were visualized by an enhanced chemiluminescence system (FluorChem Q, Proteinsimple, USA). The bands were semi-quantified using Image J software. The results were normalized to the expression level of GAPDH.

17 Hemolysis assay

Blood samples from healthy mouse were collected in a container filled with heparin, and centrifuged at 1500 rpm for 10 min to obtain red blood cells (RBCs). After washed and diluted with PBS, RBCs was mixed with HSSG and HCCG NPs dispersed PBS with different concentrations, and incubated at 37 °C. After incubation for 3 h, the mixture solutions were centrifuged, and the supernatants absorptions were measured at 570 nm (A_{sample}). Meanwhile, RBCs were mixed with PBS and water as negative control (APBS) and positive control (A_{water}), respectively. The hemolysis percentages were calculated by $(A_{sample}-A_{PBS})/(A_{water}-A_{PBS}) \times 100\%$

18 In Vivo Fluorescence Imaging.

For in vivo fluorescence imaging, H22 tumor bearing mice were first administered with IR780-HSSG NPs through intravenous injection (IR780 concentration of 10 μg kg^{-1}). The fluorescence images were obtained at different post injected times (1, 3, 6, 12 and 24 h). Subsequently, heart, liver, spleen, lung, kidney, and tumor were separated

and photographed after 24 h injection (IVIS Lumina XRMS Series III, USA).

19 In vivo antitumor efficacy

H22 tumor bearing mice were used as a model for antitumor evaluation of the GER loaded nanoparticles. When the grafted tumor reached a volume of about 150 mm^3, mice were randomly divided into 5 groups (6 mice per group) and treated with the dosages of formulation as below: saline group, HA group (15.5 mg/kg), free GER group (1.0 mg BTZ/kg), HCCG group (1.0 mg GER/kg) and HSSG group (1.0 mg GER/kg). The above formulations were injected via tail vein every 2 days, during which the mice were weighed daily, and the length (L: mm) and width (W: mm) of tumor were measured with a vernier caliper. Tumor volume (V_{Tumor}) was calculated according to the formula below:

$$V_{Tumor}=W^2\times L/2$$

20 Hematoxylin and eosin (HE) staining and Immunohistochemistry (IHC)

After 14 days treatment, all mice were sacrificed, and tumors were collected and weighted. The major organs including heart, liver, spleen, lung, kidney, and tumor were carefully excised, with 4.0% (w/v) paraformaldehyde for preservation and embedded in paraffin for further preparation of immunohistochemical sections. The specimen was cut into 7-μm thick sections and stained using Hematoxylin & Eosin. Tumor tissues were cut into 5-μm thick sections and stained using rabbit polyclonal CD31 antibody (1:200), and rabbit polyclonal anti-Ki-67 antibody (1:500). Additionally, TUNEL (transferase-mediated dUTP-biotin nick end-labeling) and immunofluorescence marker (FITC marker) were used to investigate the apoptosis of tumor cells, and the apoptosis

of tumor cells in different preparation groups was compared to evaluate and analyze the therapeutic effect in each group. The slides were observed using a fluorescence microscope.

21 Statistical analyses

Statistical analyses were performed using GraphPad Prism 8.0 software. The data are expressed as the mean ± SD. One-way ANOVA was used to determine significance of the differences using the following annotations: n.s., $*P<0.05$, $**P<0.01$, and $***P<0.001$.

References

(1) Hou, L.; Tian, C.; Chen, D.; Yuan, Y.; Yan, Y.; Huang, Q.; Zhang, H.; Zhang, Z. Investigation on vitamin e succinate based intelligent hyaluronic acid micelles for overcoming drug resistance and enhancing anticancer efficacy. *Eur J Pharm Sci* **2019**, *140*, 105071. DOI: 10.1016/j.ejps.2019.105071.

(2) Berton, P.; Shamshina, J. L.; Bica, K.; Rogers, R. D. Ionic Liquids as Fragrance Precursors: Smart Delivery Systems for Volatile Compounds. *Industrial & Engineering Chemistry Research* **2018**, *57* (47), 16069-16076. DOI: 10.1021/acs.iecr.8b02903.

(3) Wang, R.; Yang, H.; Khan, A. R.; Yang, X.; Xu, J.; Ji, J.; Zhai, G. Redox-responsive hyaluronic acid-based nanoparticles for targeted photodynamic therapy/chemotherapy against breast cancer. *J Colloid Interface Sci* **2021**, *598*, 213-228. DOI: 10.1016/j.jcis.2021.04.056.

(4) Yegappan, R.; Selvaprithiviraj, V.; Mohandas, A.; Jayakumar, R. Nano polydopamine crosslinked thiol-functionalized hyaluronic acid hydrogel for angiogenic drug delivery. *Colloids Surf B Biointerfaces* **2019**, *177*, 41-49. DOI: 10.1016/j.colsurfb.2019.01.035.

(5) Fu, D.; Ni, Z.; Wu, K.; Cheng, P.; Ji, X.; Li, G.; Shang, X. A novel redox-responsive ursolic acid polymeric prodrug delivery system for osteosarcoma therapy. *Drug Deliv* **2021**, *28* (1), 195-205. DOI: 10.1080/10717544.2020.1870583.

(6) Mu, J.; Zhong, H.; Zou, H.; Liu, T.; Yu, N.; Zhang, X.; Xu, Z.; Chen, Z.; Guo, S. Acid-sensitive PEGylated paclitaxel prodrug nanoparticles for cancer therapy: Effect of PEG length on antitumor efficacy. *J Control Release* **2020**, *326*, 265-275. DOI: 10.1016/j.jconrel.2020.07.022.

(7) Huang, Y.; Xie, D.; Gou, S.; Canup, B. S. B.; Zhang, G.; Dai, F.; Li, C.; Xiao, B. Quadruple-responsive nanoparticle-mediated targeted combination chemotherapy for metastatic breast cancer. *Nanoscale* **2021**, *13* (11), 5765-5779. DOI: 10.1039/d0nr08579k.

(8) Ye, Y.; Song, Y.; Zhuang, J.; Wang, G.; Ni, J.; Xia, W. Anticancer effects of echinacoside in hepatocellular carcinoma mouse model and HepG2 cells. *J Cell Physiol* **2019**, *234* (2), 1880-1888. DOI: 10.1002/jcp.27063.

(9) Zhang, H.; Li, W.; Guo, X.; Kong, F.; Wang, Z.; Zhu, C.; Luo, L.; Li, Q.; Yang, J.; Du, Y.; et al.

Specifically Increased Paclitaxel Release in Tumor and Synergetic Therapy by a Hyaluronic Acid-Tocopherol Nanomicelle. *ACS Appl Mater Interfaces* **2017**, *9* (24), 20385-20398. DOI: 10.1021/acsami.7b02606.

(10) Yang, Q.; Xiao, Y.; Yin, Y.; Li, G.; Peng, J. Erythrocyte Membrane-Camouflaged IR780 and DTX Coloading Polymeric Nanoparticles for Imaging-Guided Cancer Photo-Chemo Combination Therapy. *Mol Pharm* **2019**, *16* (7), 3208-3220. DOI: 10.1021/acs.molpharmaceut.9b00413.

(11) Guo, Q.; Zhang, L.; He, M.; Jiang, X.; Tian, J.; Li, Q.; Liu, Z.; Wang, L.; Sun, H. Doxorubicin-loaded natural daptomycin micelles with enhanced targeting and anti-tumor effect in vivo. *Eur J Med Chem* **2021**, *222*, 113582. DOI: 10.1016/j.ejmech.2021.113582.

(12) Gu, Z.; Wang, X.; Cheng, R.; Cheng, L.; Zhong, Z. Hyaluronic acid shell and disulfide-crosslinked core micelles for in vivo targeted delivery of bortezomib for the treatment of multiple myeloma. *Acta Biomater* **2018**, *80*, 288-295. DOI: 10.1016/j.actbio.2018.09.022.

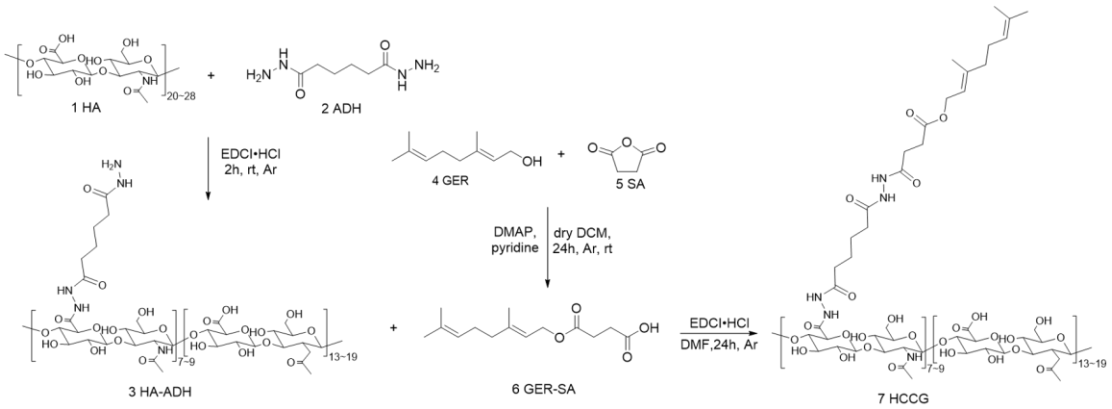

Figure S1. Synthetic pathway for the HCCG conjugate.

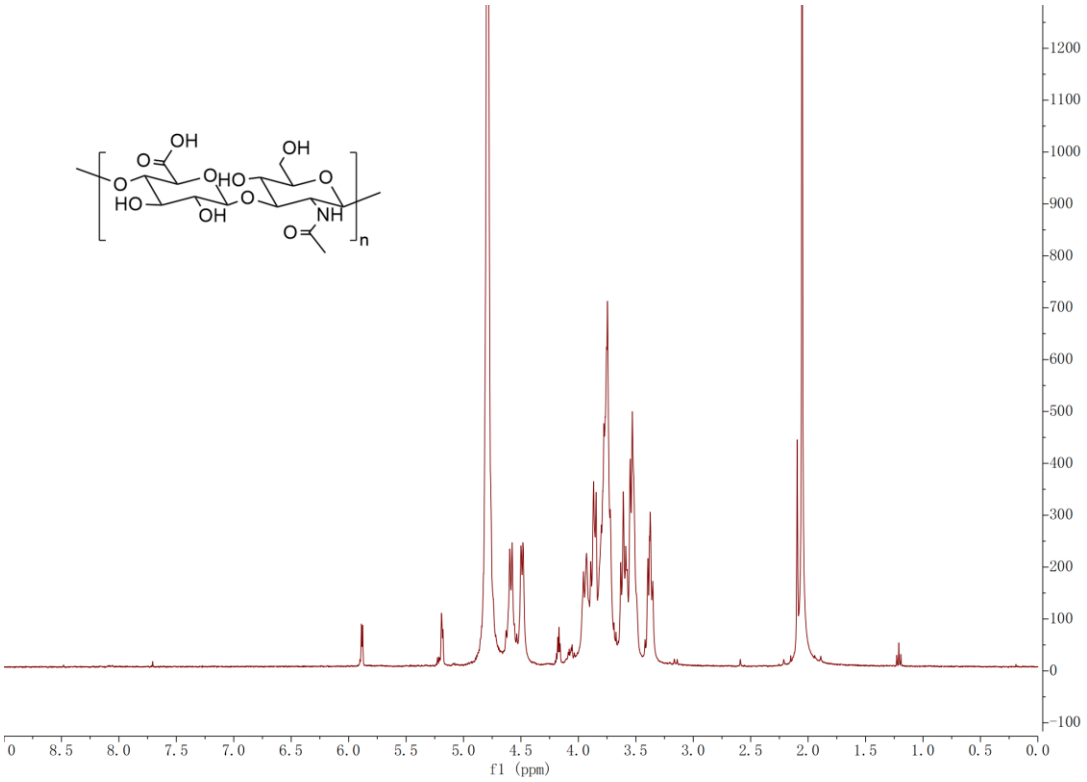

Figure S2. ^{1}H NMR spectra of HA in D_2O.

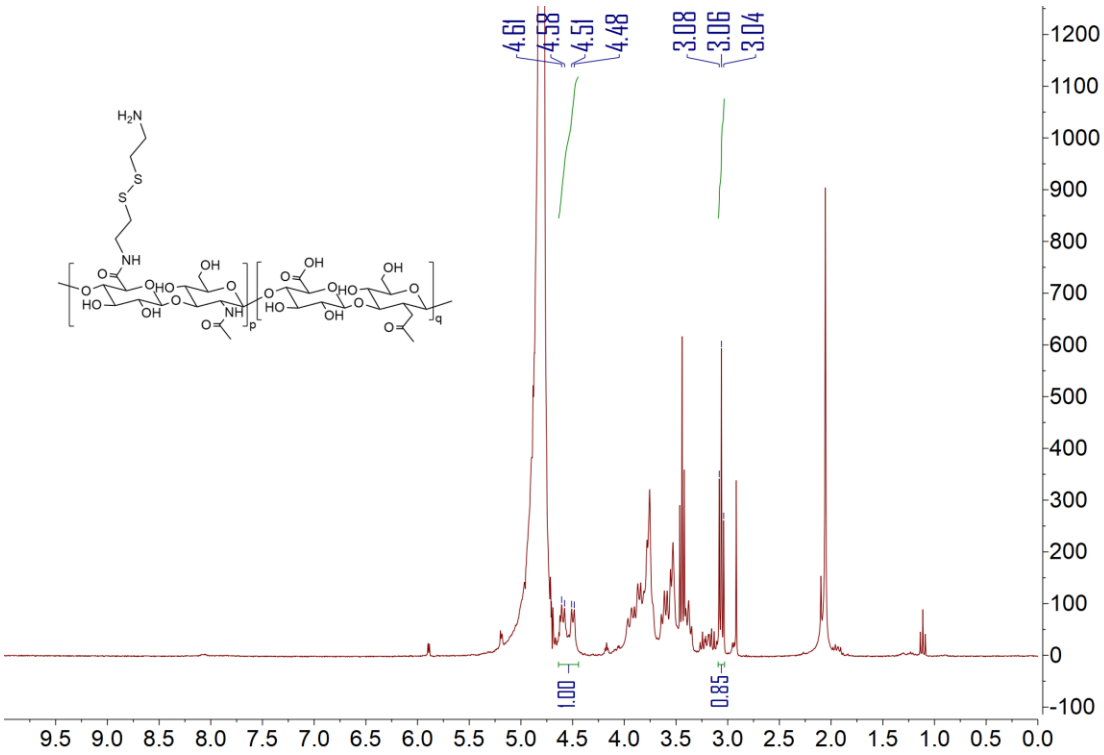

Figure S3. ^{1}H NMR spectra of HA-CYS in D_2O.

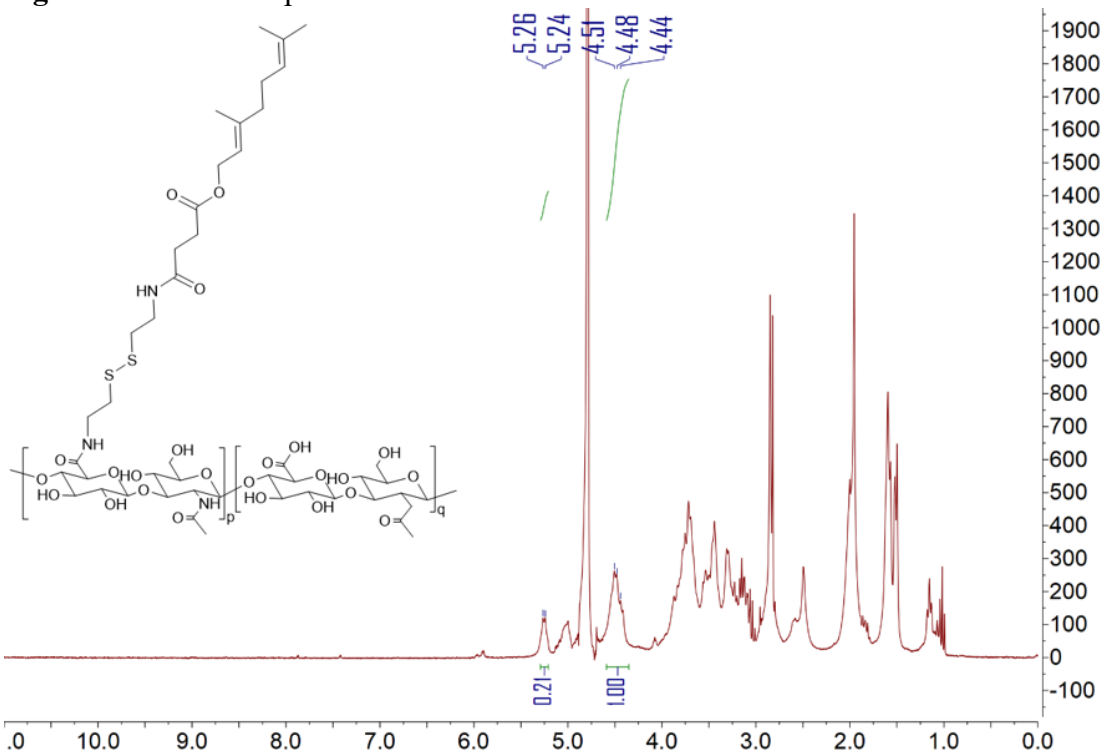

Figure S4. ^{1}H NMR spectra of HSSG in D_2O.

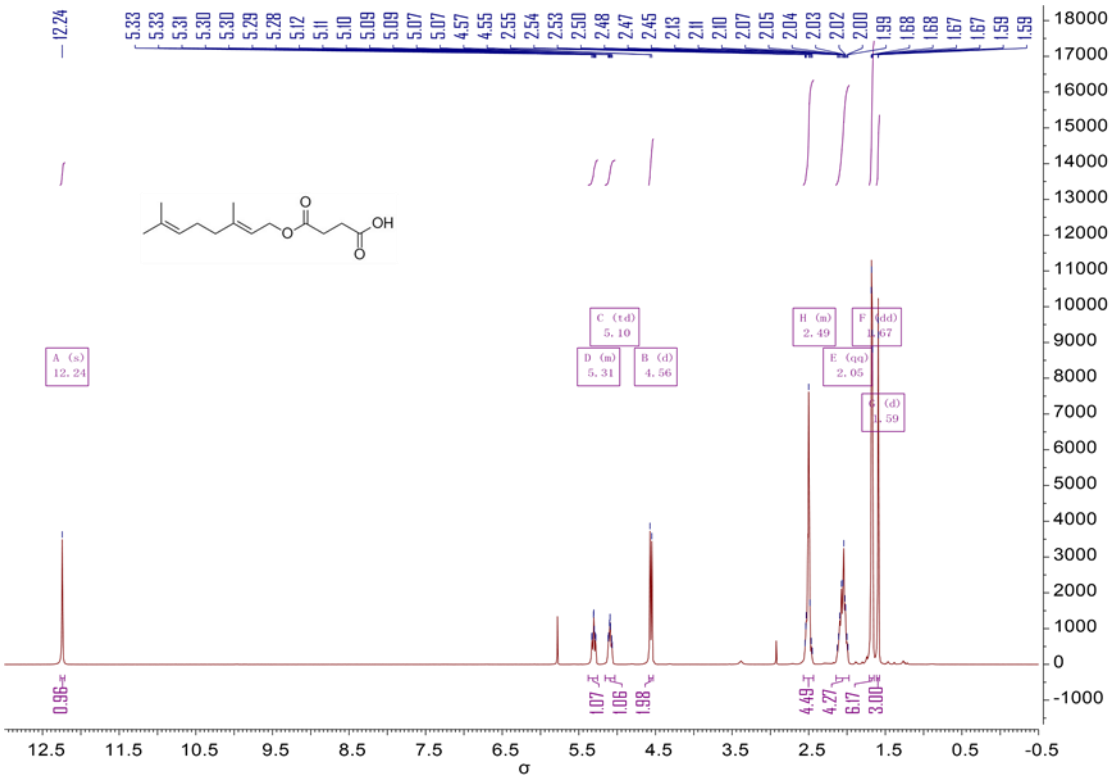

Figure S5. ^{1}H NMR spectra of GER-SA in DMSO-d_6.

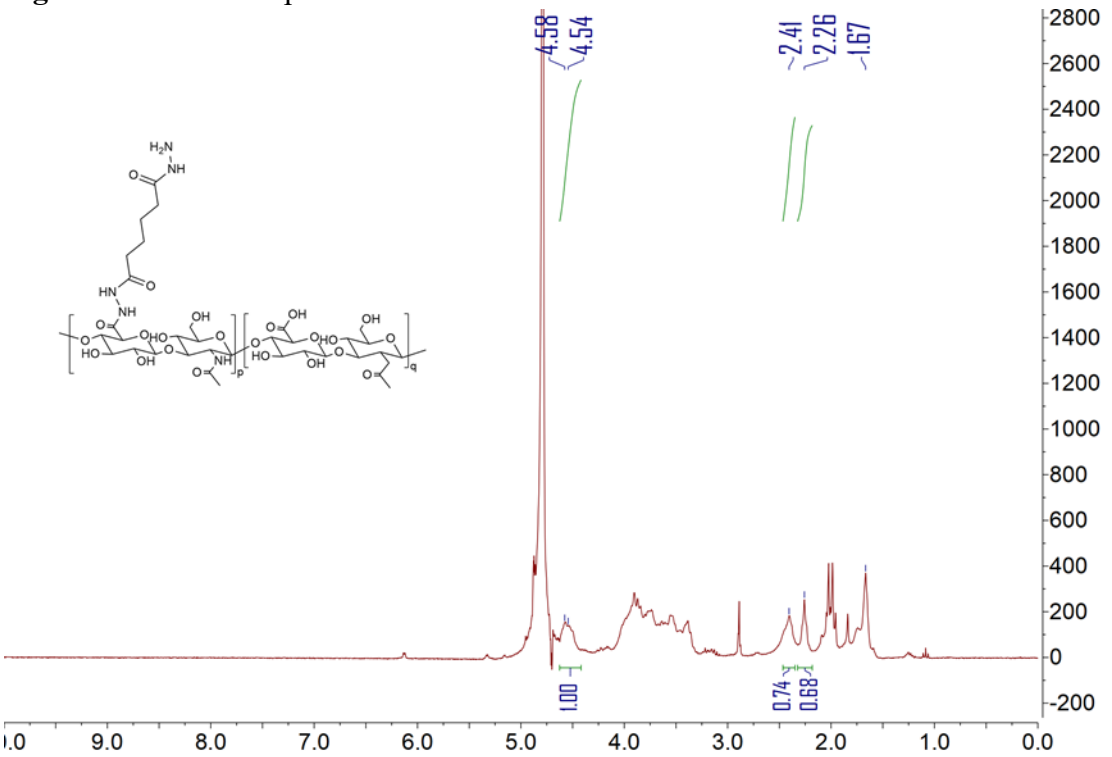

Figure S6. ^{1}H NMR spectra of HA-ADH in D_2O.

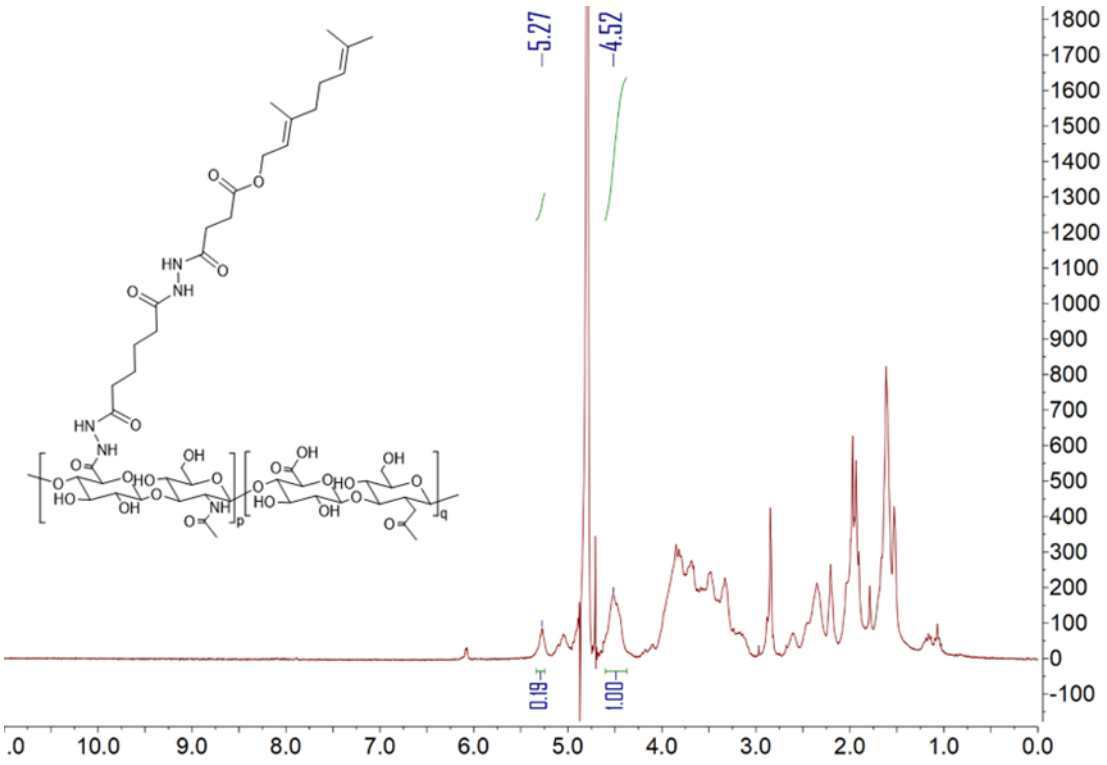

Figure S7. ^{1}H NMR spectra of HCCG in D_2O.

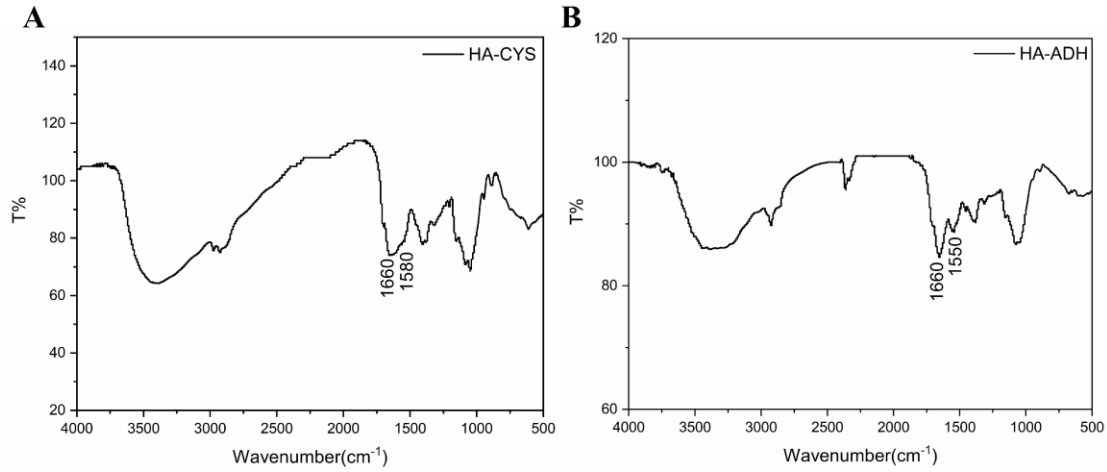

Figure S8. FT-IR spectrum of HA-CYS (A) and HA-ADH (B).

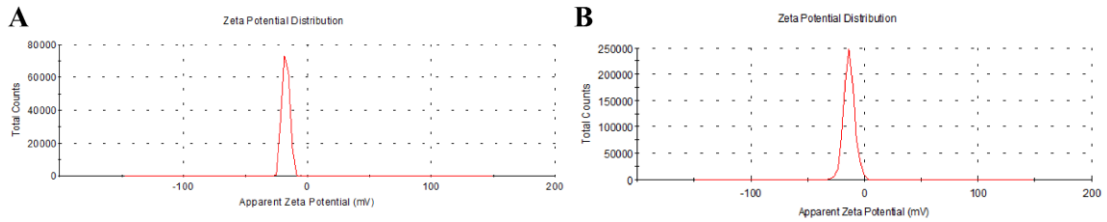

Figure S9. Zeta potential of HA-CYS (A) and HA-ADH (B).

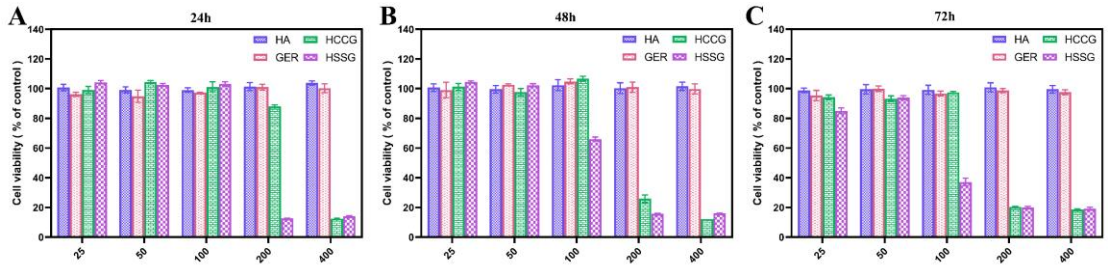

Figure S10. Cytotoxicity of HA, GER, HCCG and HSSG towards LX-2 cells for 24(A), 48(B) and 72h(C).

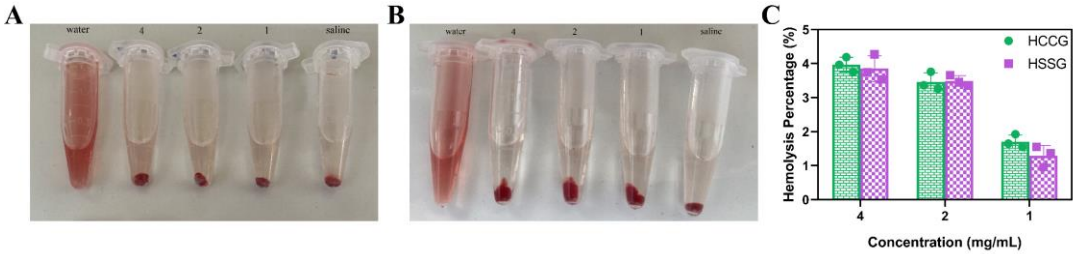

Figure S11. Hemolysis picture of HCCG (A) and HSSG (B). The hemolysis coefficient histogram of each group (C).