Supporting information for

Differentiation of Intracellular Hyaluronidase Isoform by Degradable Nanoassembly Coupled with RNA-Binding Fluorescence Amplification

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1. Materials and Instruments

RBF was obtained from our previous synthesis. Hyaluronan acid (molecular weight: 50 kDa) from bovine vitreous humor, Hyal-1, Hyal-2, cathepsin B, trypsin, thrombin, lysozyme, ribonuclease, *β*-galactosidase, and indomethacin were purchased from commercial suppliers. Cholesteryl chloroformate and 2,2'-(Ethylenedioxy)bis(ethylamine) were purchased from Aladdin Industrial Corporation. Sephadex G-50 and interleukin (IL-1) were purchased from Sigma-Aldrich. FITC-labled hyaluronidase (HAase) antibody was purchased from Shanghai Rui Qi Bio Technology Co., Ltd. RNase was purchased from Ambion Inc. (Austin, TX, USA). Human cervical carcinoma HeLa cell line (HeLa cell), human hepatoma carcinoma HepG2 cell line (HepG2 cell), and human normal hepatocyte cell line (L02 cell) were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). All chemicals were of analytical grade and used as received without further purification. All solutions were prepared using sterile ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA) and had an electric resistance >18.2 MΩ.

UV-vis absorption spectra were recorded using a Hitachi U-4100 UV/Vis spectrophotometer (Kyoto, Japan) in 1 cm path length quartz cuvettes. Zeta potential and dynamic light scattering (DLS) were obtained from Zetasizer Nano-ZS90 (Malvern Instruments, Southborough, MA) with a 15 mV solid state laser operated at a wavelength of 635 nm. Transmission electron microscopy (TEM) images were obtained with a JEOL-3010 using an accelerating voltage of 100 kV. Fourier transform infrared (FT-IR) spectra were obtained from a TENSOR 27 spectrometer (Bruker Instruments Inc., Germany). The pH was measured with a Mettler-Toledo Delta 320 pH meter. UV-B irradiation was performed by an UV-irradiator (Biosun, Vilber-Lourmat). Total RNA and DNA were extracted from HeLa cells using TRIzol (Invitrogen, Carlsbad, CA) and NP-40 (Shell Chemical Co., London, England) lysis methods according to the manufacturer’s protocol, respectively. All fluorescence measurements were performed on a PTI Fluorescence System (Photo Technology

International, Birmingham, NJ). Confocal laser scanning microscopy (CLSM) images were obtained on a Fluoview TM FV 1000 (Olympus, Japan). MTT assay was obtained on a Synergy™ 2 Multi-Mode Microplate Reader (Bio-Tek, Winooski, TV). Flow cytometric analysis was performed using flow cytometer (Gallios, Beckman Coulter Inc.).

2. Synthesis.

2.1 Synthesis of Chol-NH_2. A solution of 2,2'-(ethylenedioxy)-bis-ethylamine (EDEA, 5 g) in anhydrous dichloromethane (DCM, 30 mL) containing triethylamine (TEA, 0.75 g) was added dropwise into cholesteryl chloroformate (Chol, 3.36 g) dissolved in DCM (20 mL) over 30 min under argon. After stirred overnight at 25 °C, the final mixture was concentrated in vacuo and then purified by column chromatography (CH_2Cl_2/MeOH, 10:1) on silica gel to give the product amino-Chol Chol-HA.

2.2 Synthesis of Chol-HA. HA (1 g), dried by coevaporation with toluene in vacuo, was firstly dissolved in warm anhydrous dimethyl sulfoxide (DMSO, 25 mL) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC,25 mg), hydroxybenzotriazole (HOBT) (23 mg), TEA (20 μL) and 4-dimethylaminopyridine (DMAP, 2.5 mg) at continuous stirring under argon. After the reaction for 4 h at 25 °C, Chol-HA (100 mg) dissolved in DMSO (2.5 mL) was then added into the reaction mixture and stirred for 48 h at 25 °C to synthesize Chol-HA.

3. Examination of HA Profile.

3.1 Gel-filtration Chromatography. 1.0 mg/mL HA incubated with 100 μg/mL Hyal-1 and Hyal-2 for 3 h in PB buffer (10 mM, pH 5.0) at 37 ℃. Then, the total solutions were applied to a Sephadex G-50 column (1.5 × 120 cm) equilibrated with PBS. The column was eluted at 7 mL/h, and 3.6 ml fractions were collected. The column fractions were assayed using the ELISA-like assay for HA. The column was calibrated using HA and HA fragments (HA100: 100 saccharide units, HA6: ～6 saccharide units, HA4: ～4 saccharide units)[1].

3.2 Measurement of HA Levels. HA levels were measured using a modified method of ELISA-like assay.[2,3]

4. Evaluation of CHA Loading

The amount of RBF loaded in CHA was analyzed as follows[4]**:** 0.1 mg CHA was dispersed in 1 mL PBS buffer, and sonicated for 30 min to ensure dissolution of RBF to form RBF@CHA (nanosensor). The supernatant was then collected for fluorescence measurement after complete centrifugation of the nanosensor. The fluorescence intensity of the supernatant was determined at the excitation wavelength of 458 nm and emission wavelength of 527 nm, and the concentration of RBF were obtained from a calibration curve, which was linear over the concentration of RBF from 0.2 μM to 1.6 μM with a correlation coefficient of R^2=0.97.

Encapsulation efficiency = (weight of RBF loaded into CHA)/(initial feeding weight of RBF).

Loading content = (weight of RBF loaded into CHA)/(weight of CHA + RBF loaded into CHA).

5. Cell Incubation and Cytotoxicity Assay

5.1 Cell Culture. HeLa cell, HepG2 cell and L02 cell were maintained in Dulbecco's modified Eagle's medium (DMEM) which was containing 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin. The cells were cultured at 37 ℃ in a humidified incubator containing 5% CO_2 and 95% air. The medium was replenished every two days, and the cells were subcultured after reaching confluence.

5.2 Cytotoxicity Assay. The cytotoxicity of nanosensor was determined in cancer cell lines (HeLa) using a standard MTT assay. Briefly, 200 μL of HeLa cell suspension was seeded in flat-bottomed 96-well plates at a density of 1×10^4 cells/well and allowed to attach at 37 °C for 24 h. After rinsing with PBS, cells were incubated with 200 μL of culture media containing serial concentrations of nanosensor for 24 h, which was kept in the dark for studying the dark toxicity. The cell death induced by nanosensor was evaluated by the MTT assay according to the manufacturer's

instruction. All cytotoxicity data were obtained from parallel sample measurements (n=8) and plotted as survival percentage compared to control non-treated cells *vs* nanosensor concentrations. The cell viability was calculated with the equation: cell viability (%) = (mean OD value of treatment group/mean OD value of control) × 100%.

6. Mineralization and UV-B Irradiation

6.1 Mineralization. Cells were induced to mineralize for 6 days by the addition of 100 μM L-abscorbate-2-phosphate (AsAP), 10 mM β-glycerophosphate (β-GP) and 4 μg/ml dexamethasone (Dex) to the culture medium.

6.2 UV-B Irradiation. HeLa cells were seeded into 35 mm confocal dish (Glass Bottom Dish) at a density of 1×10^4 per dish and incubated at 37 ℃ for 24 h. Cells were washed twice with PBS. The confocal dish was irradiated with 40 mJ cm^{-2} UV-B in PBS in an UV-B irradiator at wavelength of 312 nm for 30 min. Irradiated media was eliminated and cell layer washed twice with PBS. Cells were then incubated in DMEM for another 6 h before fluorescence imaging.

7. Statistical Analysis

Data was expressed as means ± SD from at least three experiments. One-way ANOVA was used to compare the treatment effects. $P < 0.05$ was considered to be statistically significant.

8. References

1) Lokeshwar, V. B.; O¨ bek, C.; Soloway, M. S.; Block, N. L. Tumor-associated Hyaluronic Acid: A New Sensitive and Specific Urine Marker for Bladder Cancer. *Cancer Res.* **1997**, *57*, 773-777.

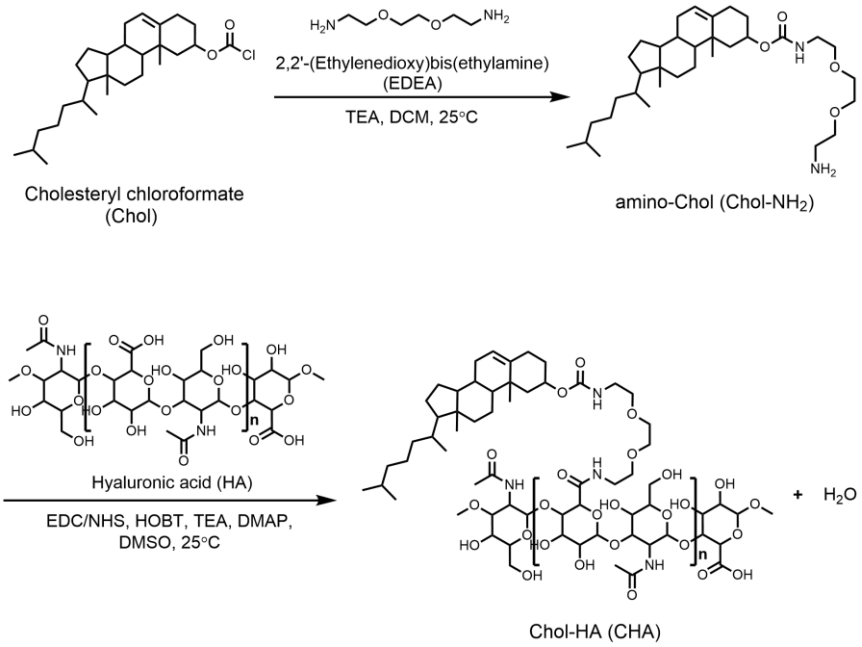
2) Fosang, A. J.; Hey, N. J.; Carney, S. L.; Hardingham, T. E. An ELISA Plate Based Assay for Hyaluronan Using Biotinylated Proteoglycan G1 Domain (HA-binding region). *Matrix* **1990**, *10*, 306-313.

3) Lokeshwar, V. B.; O¨ bek, C.; Pham, H. T.; Wei, D.; Young, M. J.; Duncan, R. C.;

Soloway, M. S.; Block, N. L. Urinary Hyaluronic Acid and Hyaluronidase: Markers for Bladder Cancer Detection and Evaluation of Grade. *J. Urol.* **2000**, *163*, 348-356.

4) Wu, M.; Wang, Q.; Liu, X.; Liu, J. Highly Efficient Loading of Doxorubicin in Prussian Blue Nanocages for Combined Photothermal/Chemotherapy Against Hepatocellular Carcinoma. *RSC. Adv.* **2015**, *5*, 30970-30980.

9. Data



Scheme S1. Synthesis route of Chol-HA.

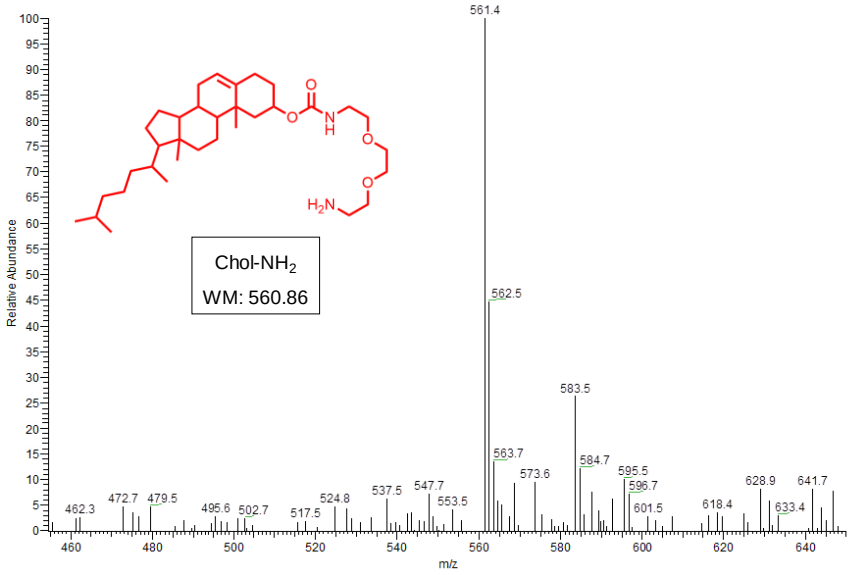


Figure S1. ESI-MS of Chol-NH_2.

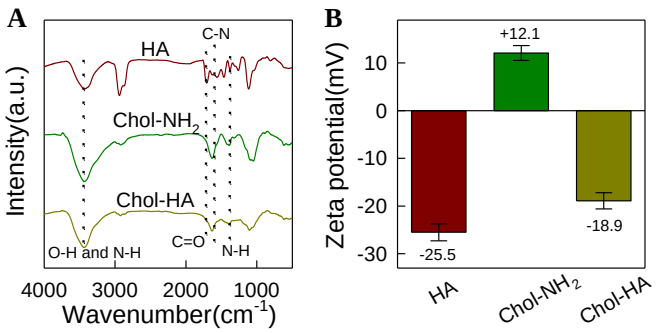


Figure S2. (A) FTIR and (B) zeta potentials of HA, Chol-NH_2, Chol-HA.

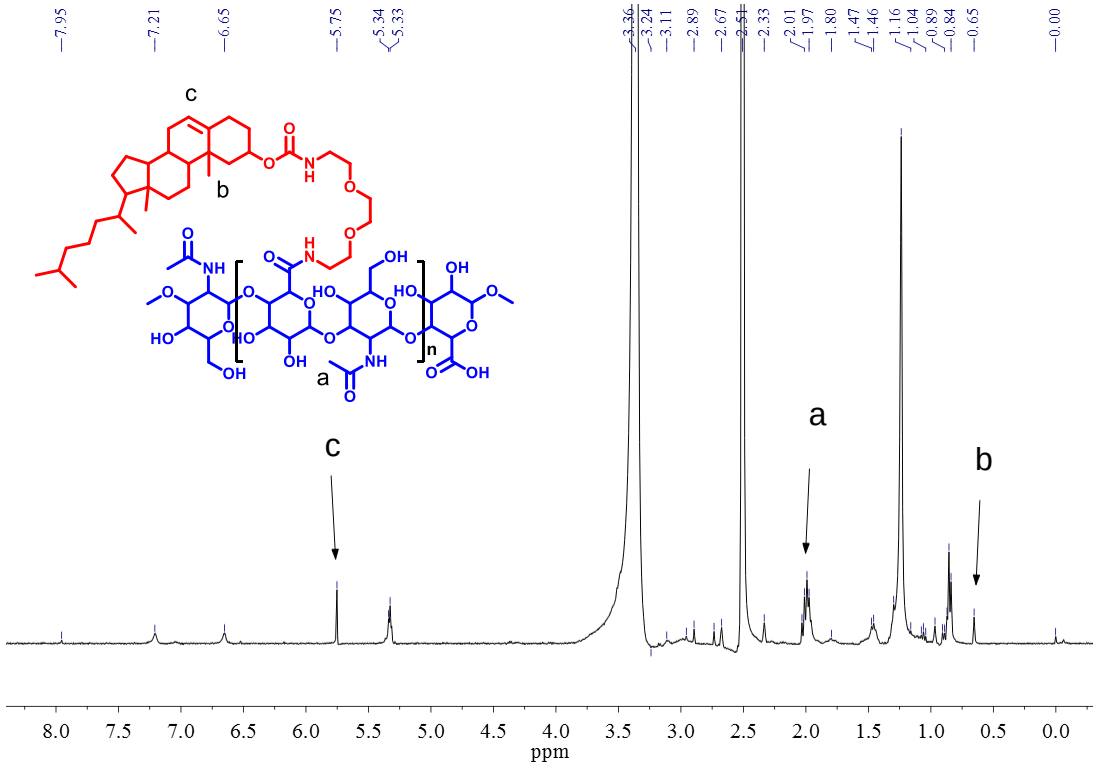


Figure S3. ^{1}H-NMR (500 MHz) spectrum of Chol-HA in DMSO-d_6. Characteristic to HA and Chol-NH_2 peaks a and b were used for calculating the substitution degrees.

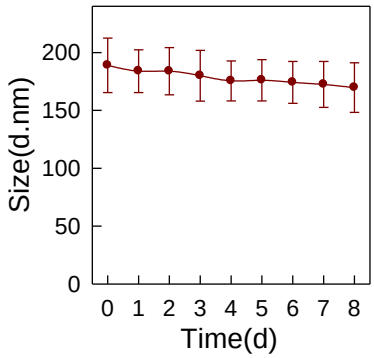


Figure S4. The average diameter fluctuation of nanosensor stored in buffer for different time periods. Error bars indicate s.d. (n=3).

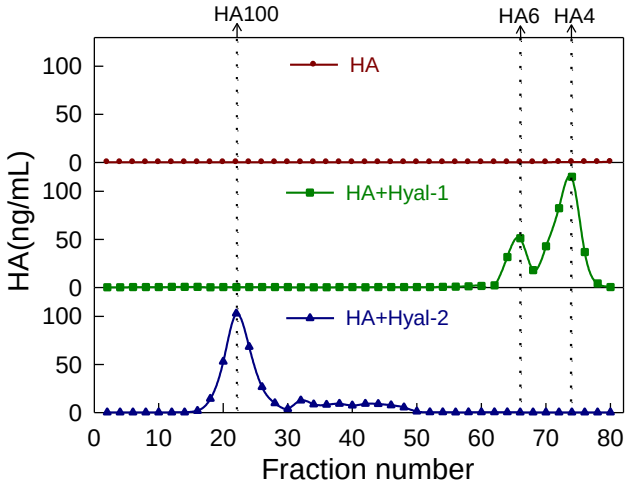


Figure S5. The size distributions of HA (1 mg) before and after Hyal-1 (100 μg/mL) or Hyal-2 (100 μg/mL) treatments determined by Sephadex G-50 gel-filtration chromatography.

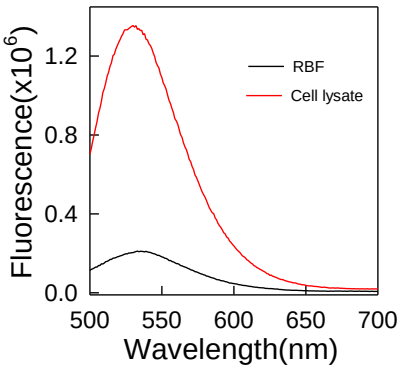


Figure S6. Fluorescence spectra of RBF (200 nM) in 10 mM phosphate buffer and cellular lysates from HeLa cells. λ_{ex} = 458 nm.

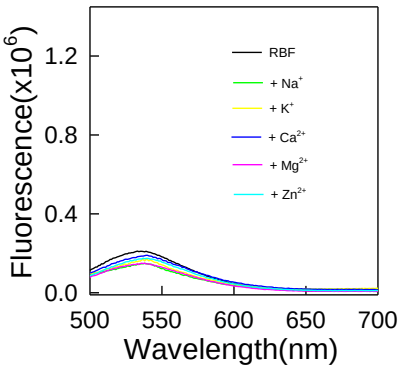


Figure S7. Fluorescence spectra of RBF (200 nM) in 10 mM phosphate buffer treated with 2.0 mM representative metal ions. λ_{ex} = 458 nm.

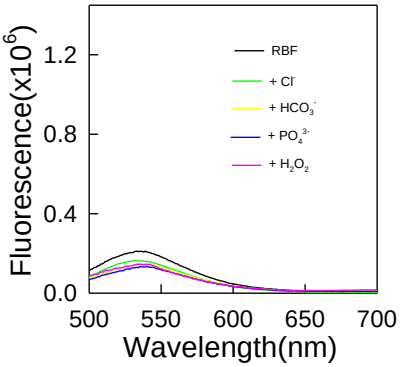


Figure S8. Fluorescence spectra of RBF (200 nM) in 10 mM phosphate buffer treated with 2 mM representative anions. λ_{ex} = 458 nm.

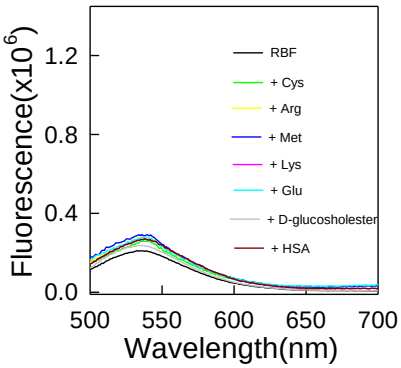


Figure S9. Fluorescence spectra of RBF (200 nM) in 10 mM phosphate buffer treated with 200 μM representative amino acids, 5.0 mM glucose, and 8.0% w/v HSA. λ_{ex} = 458 nm.

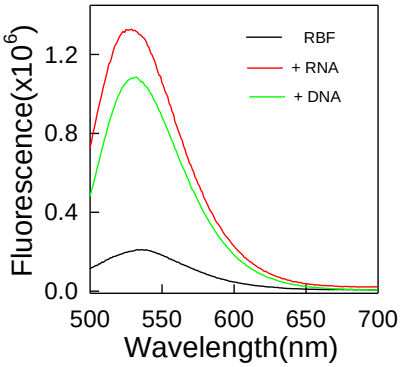


Figure S10. Fluorescence spectra of RBF (200 nM) in 10 mM phosphate buffer treated with 5 mg/mL nucleic acids. λ_{ex} = 458 nm.

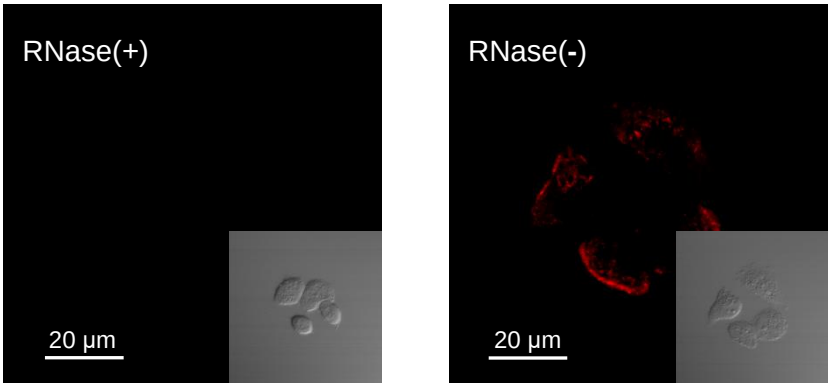


Figure S11. Fluorescence images of Hela cells incubated with RBF (2.0 μM) in the presence and absence of RNase (5 U/mL). Bar scales: 20 μm.

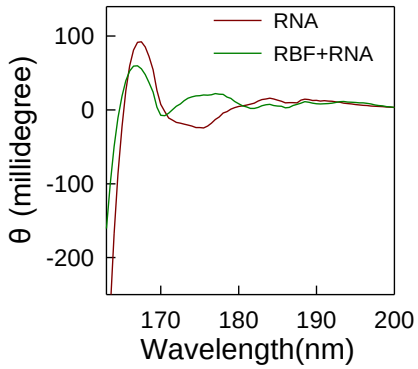


Figure S12. CD spectra of RNA (100 μg/mL) in the absence and presence of RBF (200 nM).

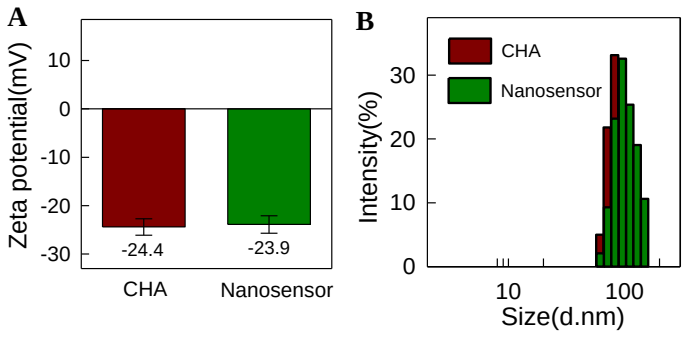


Figure S13. (A) Zeta potentials and (B) DLS of CHA and nanosensor.

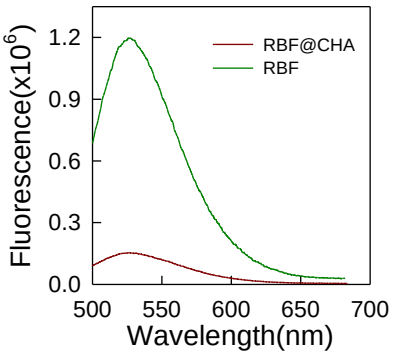


Figure S14. Fluorescence spectra of RBF(1.2 μM) and RBF@CHA(10 μg/mL).

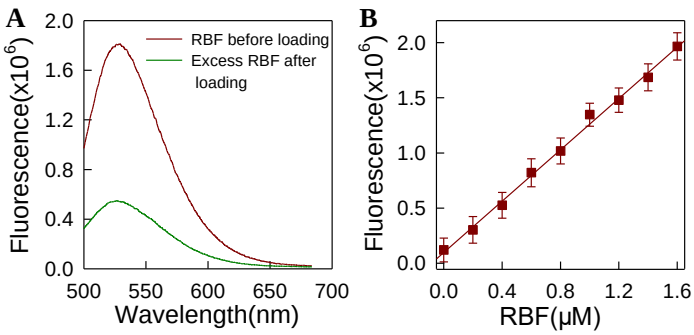


Figure S15. (A) Fluorescence spectra of RBF (1.5 μM) before and after CHA (10 μg/mL) loading. (B) The calibration curve of RBF from 0 μM to 1.6 μM. Error bars indicate s.d. (n=3).

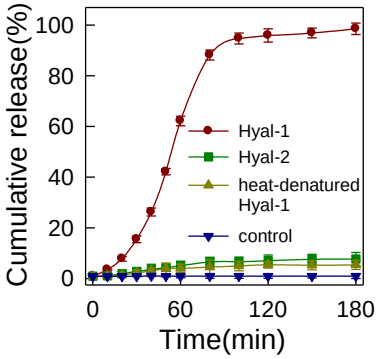


Figure S16. Cumulative release of nanosensor (2 μg/mL) before and after Hyal-1, Hyal-2, heat-denatured Hyal-1 (4 μg/mL) treatments at pH 5.0. Error bars indicate s.d. (n=3).

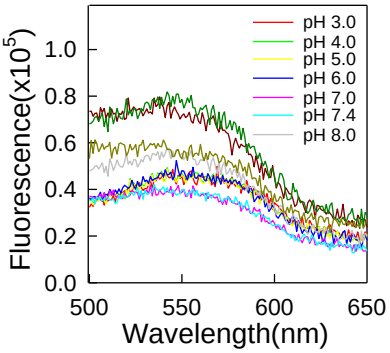


Figure S17. Fluorescence spectra of nanosensor (2 μg/mL) at different pH values.

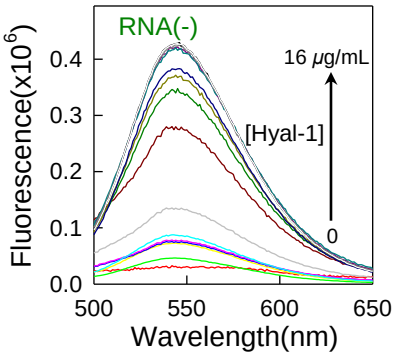


Figure S18. Fluorescence spectra of nanosensor (2 μg/mL) incubated with increasing concentrations of Hyal-1 ranging from 0 to 16 μg/mL in buffer solution.

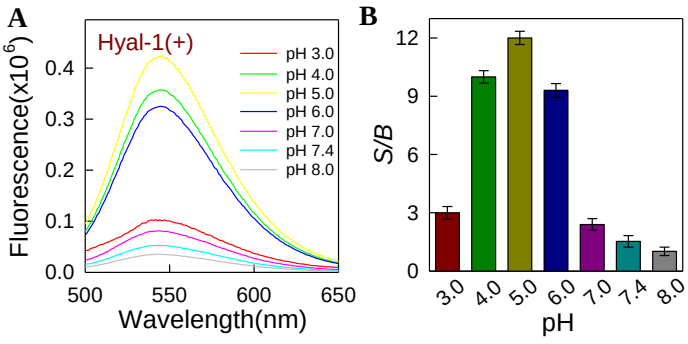


Figure S19. (A) Fluorescence spectra of nanosensor (2 μg/mL) incubated with Hyal-1 (10 μg/mL) at different pH values. (B) The corresponding fluorescence signals (S/B) of (A). Error bars indicate s.d. (n=3).

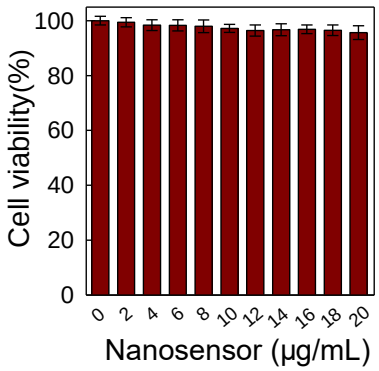


Figure S20. Cell viability of HeLa cells treated with different concentrations of nanosensor for 24 h. Error bars indicate s.d. (n=6).

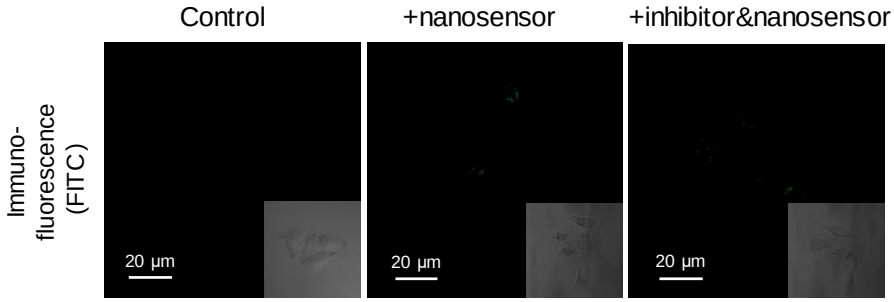


Figure S21. Fluorescence images of HeLa cells under different conditions: (left) free; (middle) incubation with FITC-labeled HAase antibody; (right) incubation with FITC-labeled HAase antibody in the presence of Hyal-1 inhibitor indomethacin. Scale bar: 20 μm.

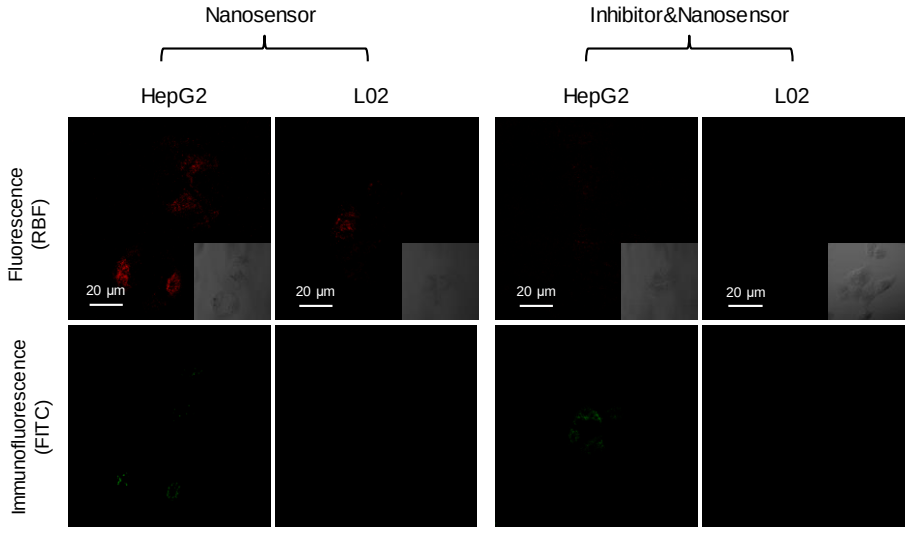


Figure S22. Fluorescence images of HepG2 and L02 cells after incubation with nanosensor (10 μg/mL) and FITC-labeled HAase antibody (1:200) in the absence and presence of Hyal-1 inhibitor indomethacin. Bar scales: 20 μm.