SUPPORTING INFORMATION

Visualizing Antithrombin-Binding 3-*O*-Sulfated Heparan Sulfate Motifs on Cell Surface

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1. Materials

Qdot 655 ITK amino (PEG) (Q21521MP) and Qdot 565 ITK amino (PEG) (Q21531MP) quantum dots were purchased from molecular probes by life technologies. Bond-breaker^{R} TCEP solution and succinimidyl-[(N-maleimidopropionamido)-dodecaethyleneglycol] ester ($SM(PEG)_{12}$) crosslinker (product code #22112) was bought from thermo scientific. Nap-10 columns (product code #17-0854-01) were bought from GE healthcare life sciences. Human antithrombin III was purchased from Cutter Biological (1000 IU/vial). Recombinant human FGF-basic (233-FB/CF) was obtained from R&D systems, Inc. Recombinant heparitinases (HepI, II and III) were expressed and purified as described.[1] The concentrations of Hep I, II and III are 1 mg/mL, 2 mg/mL and 1 mg/mL respectively. The corresponding activities of the enzymes are 723 mU/mg, 893 mU/mg and 510 mU/mg. Equal volume of the stocks containing each heparitinase isoform were mixed to prepare an enzyme cocktail for studies described here. Recombinant 3-OST-1 enzyme was expressed and purified by using established protocol.[2] 3'-phosphoadenosine-5'-phosphosulfate (PAPS) (catalog number A1551) was obtained from Sigma-Aldrich. Amicon Ultra 3000 and 50000 molecular weight cut-off (MWCO) centrifugal filters were obtained from EMD Millipore Corporation (Billerica, MA). All other chemicals were purchased from Sigma-aldrich or VWR scientific.

Human thrombin (TH) was purchased from Haematologic Technologies (Essex Junction, VT). Thrombin was stored in 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl, 0.1 mM EDTA and 0.1% (w/v) PEG8000 at -20 °C. QD655-AT was stored in 50mM borate buffer, pH 7.4 at -20 °C. Spectrozyme TH (H-*D*-hexahydrotyrosol-Ala-Arg-*p*-nitroanilide) was obtained from American Diagnostics (Greenwich, CT) and prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl, 0.1 mM EDTA and 0.1% (w/v) PEG 8000.

2. QD-AT and QD-FGF2 Bioconjugation

2.1. Quantum Dot–Protein Conjugation

QD655 (or QD565) solution (10 µL, 8 µM) was transferred to a 1.5 mL vial containing 50 mM PBS buffer, pH 7.4 (190 µL) and final volume was brought upto 200 µL. 1 mM $SM(PEG)_{12}$ (10 µL) bifunctional linker was added to the above mixture and incubated at room temperature for 2 hours and then at 4 ^{0}C for 12 hours with slow rotation (~6 rotations per minute). The resulting QD-$SM(PEG)_{12}$ was purified by filtering through Nap-10 column. Finally, the probe was centrifuged through 3000 MWCO filter and concentrated to 400 nM stock solution in 50 mM PBS buffer, pH 7.4. We tested method A and method B for protein (AT or FGF2) labeling. Both methods gave identical results. We report all our data in this paper using QD-AT or QD-FGF2 generated through method B.

Method A. TCEP Aided Conjugation[3,4]

AT (or FGF2) (dissolved in 300 µL 50 mM PBS buffer pH 7.4, 80 µM final concentration) was incubated with a 5 mM solution of TCEP (500 µL) for half an hour at room temperature to reduce S-S bridge. TCEP was then removed through ultracentrifugation using a 3000 MWCO filter at 10000 g for 10 min (x3 washed with 500 µL 50 mM PBS buffer, pH 7.4). The concentrated solution was diluted with 50 mM PBS buffer, pH 7.4 (300 µL). QD-$SM(PEG)_{12}$ (100 µL 50 mM PBS buffer pH 7.4, 400 nM) was added to the above solution and rotated in a 1.5 mL Eppendorf tube for 12 hours at 4 °C. Finally, the mixture was incubated with 0.1 mM cysteine (500 µL) for 30 min at room temperature to cap the unreacted maleimide groups of the quantum dot. QD-AT (or QD-FGF2) probe was concentrated by filtering through 50000 MWCO filter (x3 washed with 500 µL 50 mM PBS buffer, pH 7.4)

Method B. Thiolate-Disulfide Exchange[5,6,7]

QD-SM(PEG)$_{12}$ (100 µL, 400 nM) was treated with AT (or FGF2) (300 µL, 80 µM) solutions in 50 mM PBS buffer, pH 7.4 in presence of 1 mM cysteine (500 µL) for 12 hours at 4 °C. The mixture was kept at room temperature for 30 min to enhance the rate of thiolate-disulfide exchange and deactivation of free maleimide groups on QDs. The final mixture was concentrated through 100000 MWCO filter (x3 washed with 500 µL 50 mM PBS buffer, pH 7.4). Both QD-AT and QD-FGF2 were stored at a concentration of 400 nM in 50 mM PBS buffer, pH 7.4 at 4 °C and used for experiment within two weeks of preparation.

2.2. Agarose Gel Electrophoresis

A mobility shift between QD655 and QD-AT in a 1% agarose gel electrophoresis (AGE) (0.6 g) in 1X TBE buffer, pH 8.3 (60 mL) (**Figure S2A**, **Lane 1, 2**).[8,9] The gel was run for 2 hours at 50 volt and imaged in Typhoon optical scanner (Molecular Dynamic Inc) by using 670 nm bandpass filter (670 DF 30 for QD655) and 526 nm short-pass filter (for FITC). The probe was also incubated with FITC labeled heparin, a heterogeneous 3-*O*-sulfated glycosaminoglycan and then allowed to run in 1% AGE (**Figure S2A, Lane 3**). A streak of FITC labeled heparin suggests the binding between QD-AT and FITC-heparin. This smear was also observed when FITC-heparin was incubated with AT only (**Figure S2A, Lane 4**). In contrast, FITC-heparin alone shows only single band (**Figure S2A, Lane 5**). The results suggest that QD-AT probe is efficient in binding with 3-*O*-sulfated pentasaccharide residue of heparin and thus, the QD-AT probe can be used to visualize anticoagulantly active HSPG structures. A similar AGE mobility shift assay between QD and QD-FGF2 confirmed the synthesis of the probe (**Figure S2B)**.

2.3. Mobility Shift Analysis

To analyze the structural integrity of FGF2 following QD labeling, we studied the interaction between QD-FGF2 and FITC-labeled heparin through 1% agarose gel electrophoresis (**Figure S2C**). The three lanes include (lane 1) QD-FGF2 incubated with FITC-heparin (1 h at RT or 16 h at 4 °C); (lane 2) FGF2 incubated with FITC-heparin (1 h at RT 16 h at 4 °C) and (lane 3) FITC-heparin. Shifts in mobility of QD-FGF2 and FGF2 are clearly observed, in contrast to that of FITC-heparin that migrates all the way to the bottom. Such shits in mobility indicate high-affinity interaction for the two FGF2 species with heparin,[10] QD-FGF2/FITC-heparin (lane 1) showed a slightly diminished signal of FITC at either 565 nm (QD fluorescence) or 520 nm (FITC fluorescence) presumably due to the FRET between FITC and QD. This suggests a strong binding between QD-FGF2 and FITC-heparin analytes.[11,12] We also plotted the intensities across each lane (λ_{EM} = 565 nm for QD-FGF2 and 520 nm for FITC) (**Figure S2D**). Lanes 1 and 2 showed a similar trend in mobility shift towards high molecular weight in contrast to FITC-heparin (lane 3). This suggests the maintenance of structure around the heparin-binding site of FGF2 following QD conjugation.

3. Biophysical Studies

3.1. Equilibrium Binding Studies using Fluorescence Spectroscopy

The equilibrium dissociation constants of heparin pentasaccharide (H5)–(QD-AT) complexes were measured using change in fluorescence emission as a function of the concentration of H5 in 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl, 0.1 mM EDTA and 0.1% PEG8000 at 25 °C, as described earlier.[13,14] The experiments were performed using a QM4 fluorometer (Photon Technology International, Birmingham, NJ) in a quartz microcuvette by titrating a 200 µL solution of the QD-AT (5-20 nM) and monitoring the change in the fluorescence at 340 nm (λ_{EX} = 280 nm). Excitation and emission slit width were set to 1.0 mm for each experiment. The saturable change in fluorescence signal was fitted using the quadratic equilibrium binding equation 1 to obtain the K_D of interaction. In this equation, ΔF represents the change in fluorescence at a fixed concentration of H5 from the initial fluorescence F_0 and ΔF_{MAX} represents the maximal change in fluorescence

following saturation of the protein. $[P]_0$ represents the total concentration of QD-AT.

$$\frac{\Delta F}{F_0} = \frac{\Delta F_{max}}{[QD-AT]_0} \times \left\{\frac{([QD-AT]_0 + [H5]_0 + K_D) - \sqrt{(([QD-AT]_0 + [H5]_0 + K_D)^2 - 4[QD-AT]_0[H5]_0)}}{2}\right\}$$

3.2. Stoichiometric studies using enzyme kinetics

The kinetics of inhibition of coagulation proteases TH, by QD-AT was measured spectrophotometrically using a microplate reader (FlexStation III, Molecular Devices) under pseudo-first-order conditions, as described earlier.[15] Briefly, a fixed concentration of QD-AT (x nM) was incubated with six varying concentrations of TH (0.5 – 25 nM) in 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl, 0.1 mM EDTA and 0.1% (w/v) PEG 8000 at 25 °C. The inhibition reaction was allowed to incubate overnight and the reaction mixture was quenched with 100 µL of 125–200 µM chromogenic substrate (Spectrozyme TH) in 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl at 25 °C. To determine the slope, the rate of substrate hydrolysis was measured from the increase in absorbance at 405 nm. The protease activity at each concentration of TH was calculated using the slope and the profile was fitted on a plot of slope vs [QD-AT/TH], which was used to determine the stoichiometry of the reaction.

4. Cell Culture

4.1. CHO-K1

0.05 X 10^6 cells per well were seeded in a 12-well plate with 15 mm microscope cover glass (Fisherbrand, 12-545-83) for 24 hours at 37 ^{0}C. Ham F-12 (X1) nutrient mixture was used.

4.2. BLMVEC

0.05 X 10^6 cells per well were seeded in a 12-well plate with 15 mm microscope cover glass (Fisherbrand, 12-545-83) for 24 hours at 37 ^{0}C. MCDB-131 complete (with fetal bovine serum and antibiotics, MCDB-131 10) endothelial media was used.

5. Cell Labeling

5.1. Sample Preparation

The samples of desired concentration were prepared by diluting 50 mM PBS buffer (pH 7.4) to a 400 nM stock solution of QD-AT or QD-FGF2.

5.2. Labeling

The media was pulled from the cells grown on cover glass in a 12-well plate. The cells were then washed with 1XPBS buffer, pH 7.4 (3X 500 µL), fixed with 4% paraformaldehyde for 20 minutes, and incubated with QD-AT or QD-FGF2 (15 nM, 300 µL) for 2 h at room temperature while rocking on a shaker. The probes were removed and cells were washed with 1X PBS buffer (3X 500 µL). The QD-AT or QD-FGF2 labeled CHO-K1 and BLMVEC cells were further labeled with actin (phalloidin-488, see section 8.1) or anti-heparan sulfate antibody (10E4, see section 7.2.1). The experiments were carried out in triplicates.

6. Cell Treatment Experiments

6.1. 3-OST-1/PAPS Modification of Fixed Cells

In order to modify cell surface GAG of three wells of a 12-well plate, 3-OST-1/PAPS *cocktail* was prepared by mixing a) 3-OST-1 (100 µL, 1 µg/µL), b) PAPS (200 µL, 5 µg/µL), c) 5X MES buffer (500 µL) and d) autoclaved water (1000 µL). The cells were first fixed with 4% paraformaldehyde (PFA) for 10 minutes, washed with 1X PBS buffer, pH7.4 (3X 500 µL). The cell surfaces were then modified with 3-OST-1 enzyme/PAPS (600 µL of *enzyme cocktail* per well) for 72 hours at 37 ^{0}C. Autoclaved water (200 µL) was added to each well every 24 hours in order to preserve the enzyme cocktail concentration. After 3 days of incubation, the 3-OST-1/PAPS was pulled off from each well, washed with 1X PBS buffer three times and treated with QD-AT (15 nM, 300 µL). The QD-AT treated cells were placed on standard analog shaker for 2 hours at room temperature. Finally, the QD-AT probe was removed from the wells and cells were washed *gently* with 1X PBS buffer, pH 7.4 three times. Fluorescence microscopy was used to image the fixed cells labeled with QD-AT.

6.2. Heparitinase Treatment

In order to treat cells with hep I, II, III enzymes, the enzyme cocktail (10 µL per well of a 12-well plate) in 10X hep buffer (1 µL per well of a 12-well plate) and appropriate media (289 µL) were added to each well and kept at 37 ^{0}C for 3 hours.[9] The media was then pulled, cells were washed with 1X PBS buffer, pH 7.4 three times (3 X 500 µL) and fixed with 4% paraformaldehyde (PFA) for 10 minutes. The cells were again washed with 1X PBS buffer, pH 7.4 three times (3 X 500 µL). Finally, the hep I, II, III treated cells were labeled with QD-AT (or QD-FGF2) (15 nM, 300 µL) at room temperature for 2 hours. The cells were *gently* washed with 1X PBS buffer, pH 7.4 three times (3 X 500 µL) and imaged by using fluorescence microscopy.

7. Cell staining protocols

7.1. Actin staining

QD-AT labeled cells were treated with 10% goat serum and 0.1% triton-X for an hour at room temperature, washed with PBS buffer once and then incubated with DAPI (1:2500 dilution) and phalloidin-488 (1:1000 dilution) at room temperature for 2 hours. The cells were washed with PBS buffer three times and mounted on glass slide for imaging.

7.2. Antibody Labeling

7.2.1. 10E4 labeling (CHO-K1, BLMVEC)

Cells (QD-AT or QD-FGF2 labeled) were blocked with 10% goat serum and 0.05% triton-X for 1 hour at room temperature. Next, the cells were incubated with primary antibody overnight at 4 ^{0}C. (10E4 dilution- 1:50 in 1% goat serum), washed three times with PBS buffer, 10 min each time while shaking on a shaker, incubated with secondary antibody for 1 h at room temperature (goat anti-mouse IgM-488 dilution- 1:750 in 1% goat serum), washed three times with PBS buffer, 10 min each time while shaking on a shaker, incubated with DAPI (1:2500 dilution in PBS) for 20 min, washed two times with PBS buffer, 5 min each time while shaking on a shaker, fixed with 4% PFA for 20 min, washed two times with PBS buffer, 5 min each time while shaking on a shaker. Finally, the 10E4- labeled cells were mounted on a glass slide for confocal imaging.

8. Mouse Aorta Labeling

Two weeks old (N=3) and six months old mice (N=3) aorta were surgically removed and cut into about 2 mm rings. The aorta rings were divided into two groups—a) QD-AT treated and b) heparitinase/QD-AT treated aorta. For group b), hep I, II, III enzymes (30 µL) was prepared in F12/DMEM (1:1) media (250 µL). The aorta rings were incubated with this enzyme for 2 hours at 37 ^{0}C on a rotor (5% CO_2). The cap of the tube was kept half open for CO_2 gas to enter the tube. The aorta rings were then washed with HBSS buffer three times, F12/DMEM (1:1) media once and

then treated with QD-AT probe. For groups a) and b), QD-AT (30 nM) in F12/DMEM (1:1) media (250 μL each) was added to mouse aorta and for 2 hours at 37 ^{0}C (5% CO_2) on a rotor. The aorta rings were washed with HBSS buffer three times, fixed in 4% PFA overnight at 4 ^{0}C. Next day, 4% PFA was exchanged with PBS buffer.The rings were then prepared for cryosection as follows- one ring from each group was put into 10% sucrose solution at 4 ^{0}C for an hour (until the ring settled down at the bottom). Next, 10% sucrose solution was replaced by 30% sucrose solution at 4 ^{0}C and finally cryosection was performed. The cryosection glass slides were washed gently in PBS buffer, pH7.4 twice for 5 min. The sections were then treated with DAPI for 30 min, air-dried, applied fluoromount and sealed for confocal microscopy.

9. Confocal Laser Scanning Microscopy

Fluorescent images were collected with a Nikon A1 microscope using Plan Fluor 40X Oil DIC H N2 and Apo 60× Oil λS DIC N2 objectives. The confocal aperture was set to one airy unit, and these settings were maintained for all the samples. DAPI, phalloidin 488 (10E4 and Tau-MAPT+ antibody labeling), QD-FGF2 and QD-AT were excited using a 405 nm (emission= 450 nm), 488 nm (emission= 525 nm), and 561 nm (emission= 595 nm) and 638 nm (emission= 700 nm) laser lines respectively. Acquisition software used was NIS-elements AR 4.11.00 64-bit. Images were analyzed using NIH ImageJ for quantitative analysis of neuronal morphology using segmented line and angle tools. Each experiment was performed in triplicate.

10. Statistical Analysis

All statistical analysis was performed using Fiji, Microsoft excel and Prism 8.2. Four data sets of 40X magnification were acquired for all *in vitro* plots and statistical analysis (N= 4). For the aorta data analysis, 20X images of 4 cryosections (N=3 mice in each age group) were used. Fiji app was used to generate QD-AT or QD-FGF2 probe's integrated density, which is the sum of the all pixel brightness values. Data were analyzed using an unpaired two-tailed Student's t-test. All graphs are depicted with error bars corresponding to the standard error of the mean.

11. FIGURES

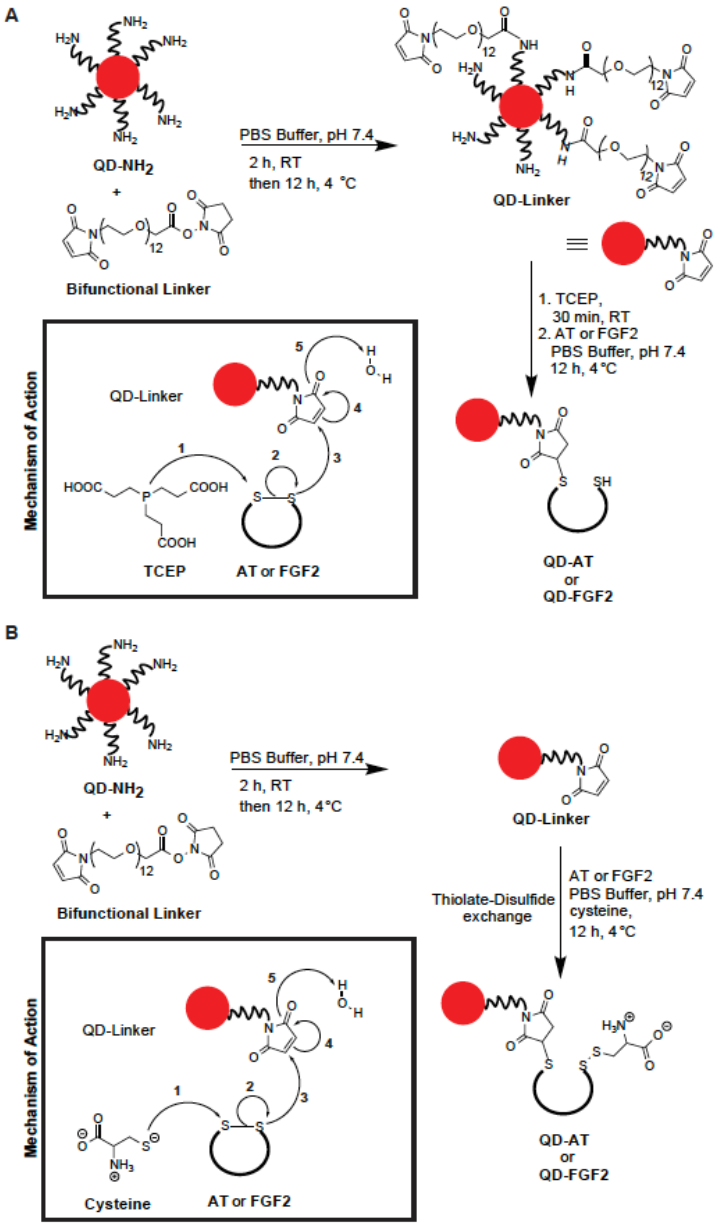

Figure S1. QD-AT and QD-FGF2 bioconjugation. The first step involves nucleophilic addition-elimination reaction between amine group of the quantum dot and the N-hydroxysuccinimidyl group of the bifunctional linker to make an amide bond between QD and the linker. **A)** TCEP method and **B)** thiolate-disulfide exchange involve **1, 2** = nucleophilic S_N2 substitution; **3, 4**= thio Michael addition; **5**= proton transfer step

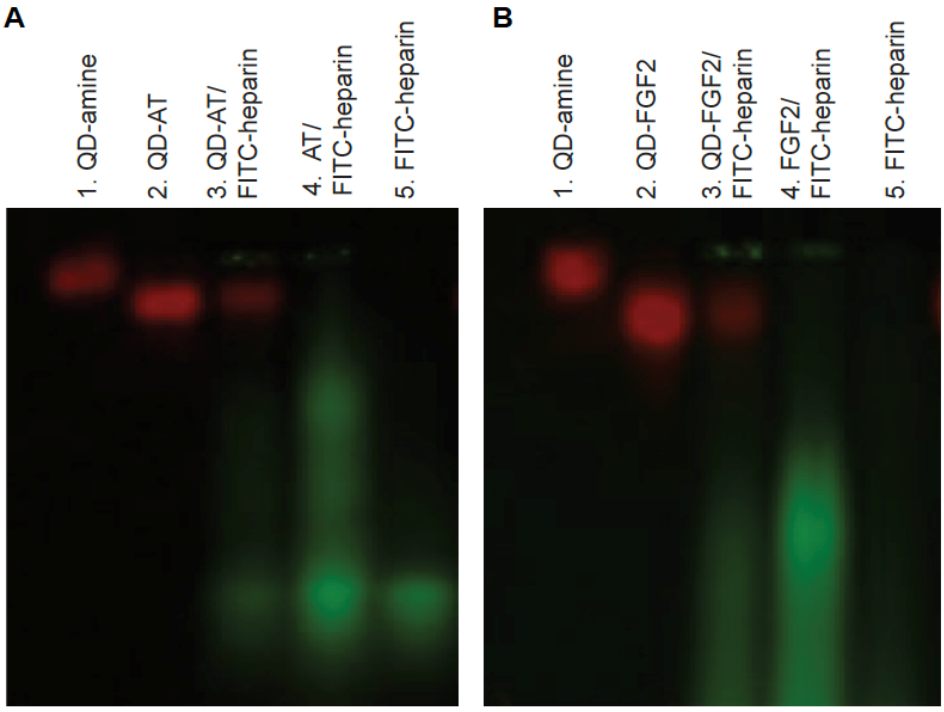

Figure S2. Agarose gel electrophoresis of **A)** QD v. QD-AT (red color) and **B)** QD v. QD-FGF2 (red color) incubated with FITC conjugated heparin (green color) shows electrophoretic mobility shift between QD-AT (or QD-FGF2) alone (**lane 2**) and QD-AT (or QD-FGF2) incubated FITC-heparin (**lane 3**).

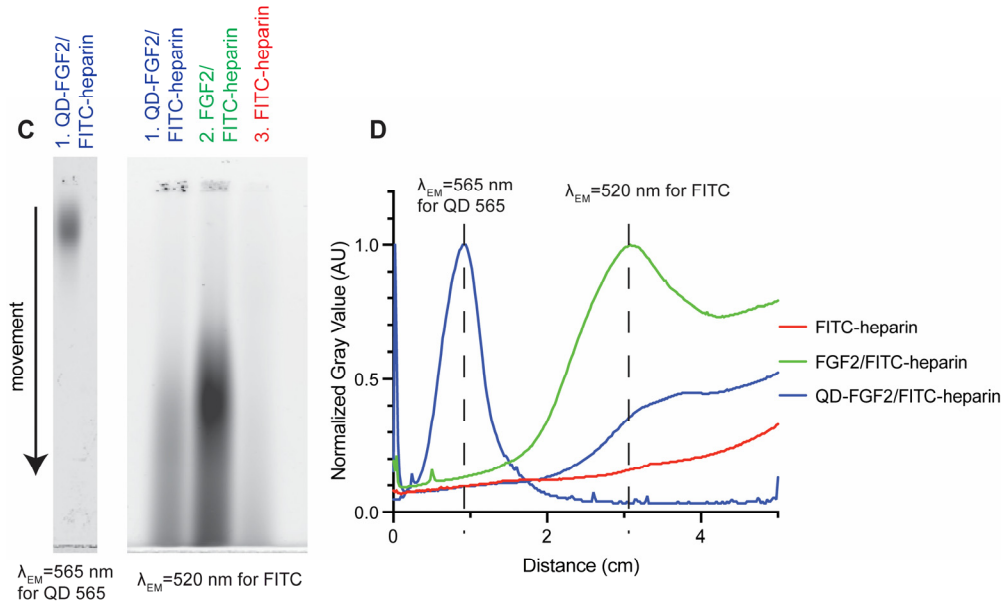

Figure S2. Mobility shift analysis. C) QD-FGF2 incubated with FITC-heparin (lane 1) and imaged at λ_{EM} = 565 nm for QD and λ_{EM} = 520 nm for FITC. Likewise, FGF2 incubated with FITC-heparin (lane 2) imaged at λ_{EM} = 520 nm for FITC. FITC-heparin (lane 3) imaged at λ_{EM} = 520 nm for FITC. **D)** Plot of the normalized intensity values as a function of distance (cm). A mobility shift between lane 1 (and 2) and lane 3 represents FGF2 interaction with heparin. FIJI software is used to generate the intensity values by drawing a line across each lane, normalized the values in excel, and plot the graph in Prism.

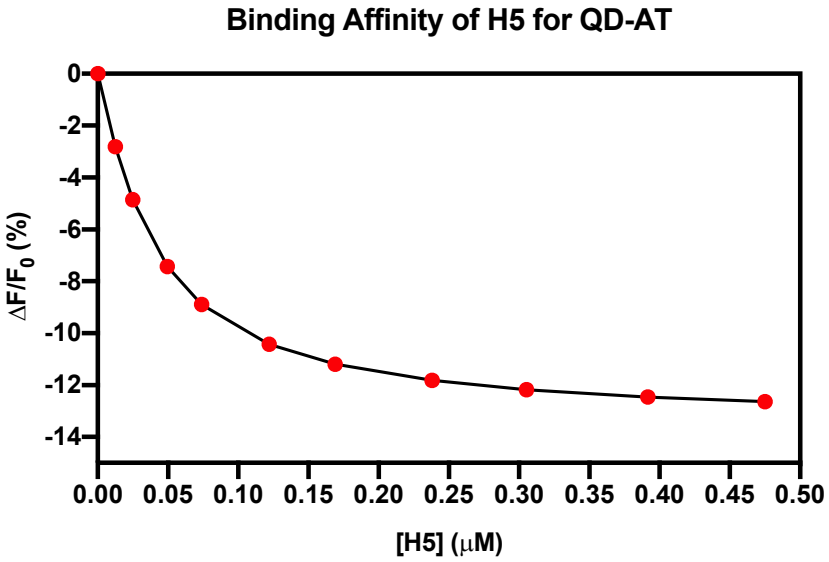

Figure S3. Affinity of heparin pentasaccharide H5 for QD-AT in 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl at 25 °C. The affinity was measured from the proportional change in fluorescence emission ($\Delta F/F_0$) at 340 nm (λ_{EX} = 280 nm) as a function of the concentration of the H5, which was fitted using quadratic equation 1 (solid line) to derive the maximal fluorescence change (ΔF_{MAX}) and equilibrium dissociation constant (K_D). See section 3.1 for additional details.

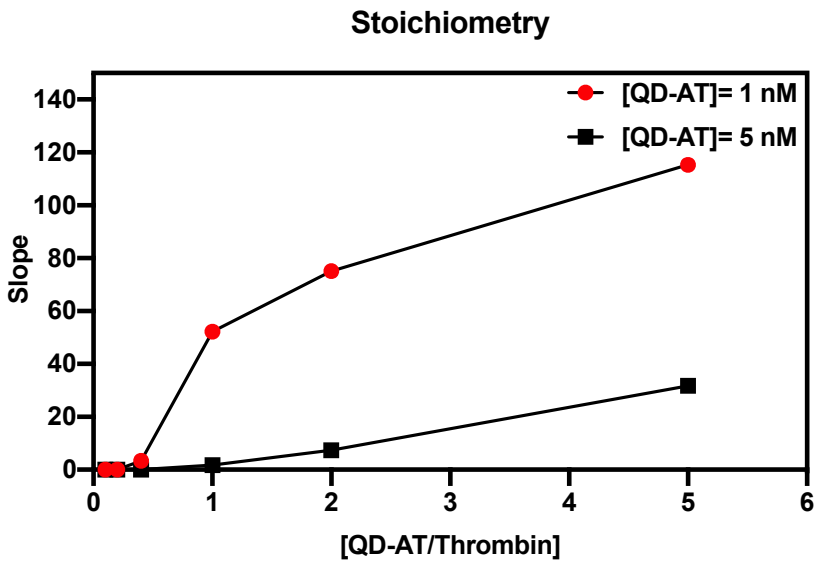

Figure S4. Kinetics of QD-AT inhibition of TH in 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl at 25 °C. The slope was measured from the rate of substrate hydrolysis at fixed concentrations of QD-AT (1 and 5nM). The profile of the rate of substrate hydrolysis (slope) at different TH concentrations was plotted as a ratio of [QD-AT:TH] and its analysis was used to obtain the stoichiometry of the reaction.

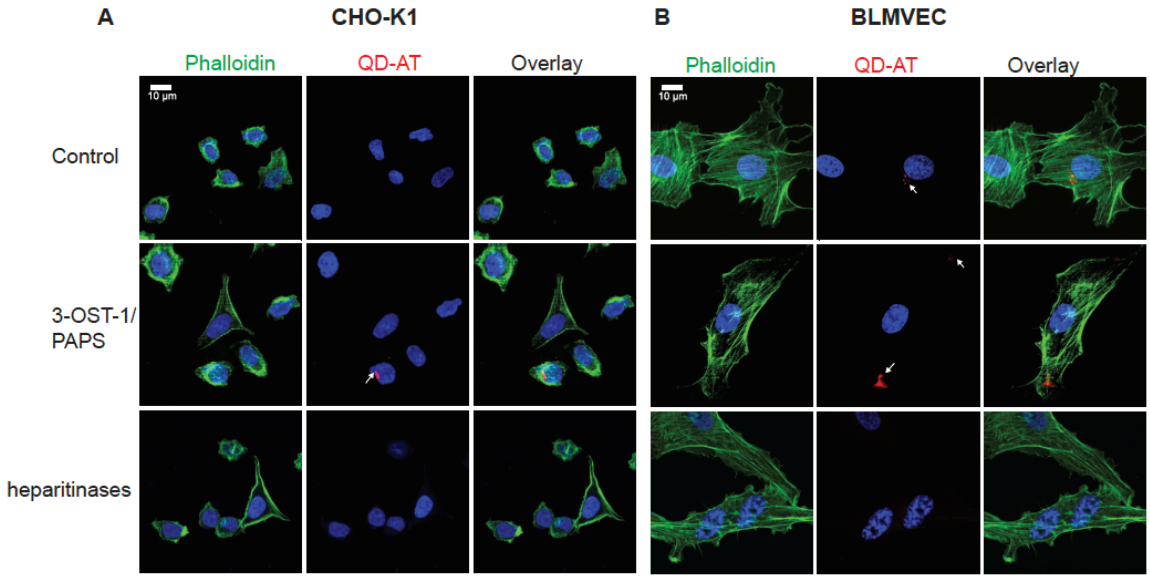

Figure S5. QD-AT labeling in A) Chinese hamster overy-K1 (CHO-K1) cells and **B**) bovine lung microvascular endothelial cells (BLMVECs). QD-AT (red) detects heparin-like 3-*O*-sulfated heparan sulfate (HS) pentasaccharide. 3-O-sulfotransferase-1 (3-OST1) enzyme and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) cocktail produces new 3-*OS*-HS epitopes as identified by QD-AT probe. Heparitinase enzyme treatment digests the 3-*OS*-HS epitopes and abolishes the fluorescent signal. Phalloidin staining (green) labels the actin cytoskeleton. (60x magnification, Scale bar = 10 µm, blue= DAPI)

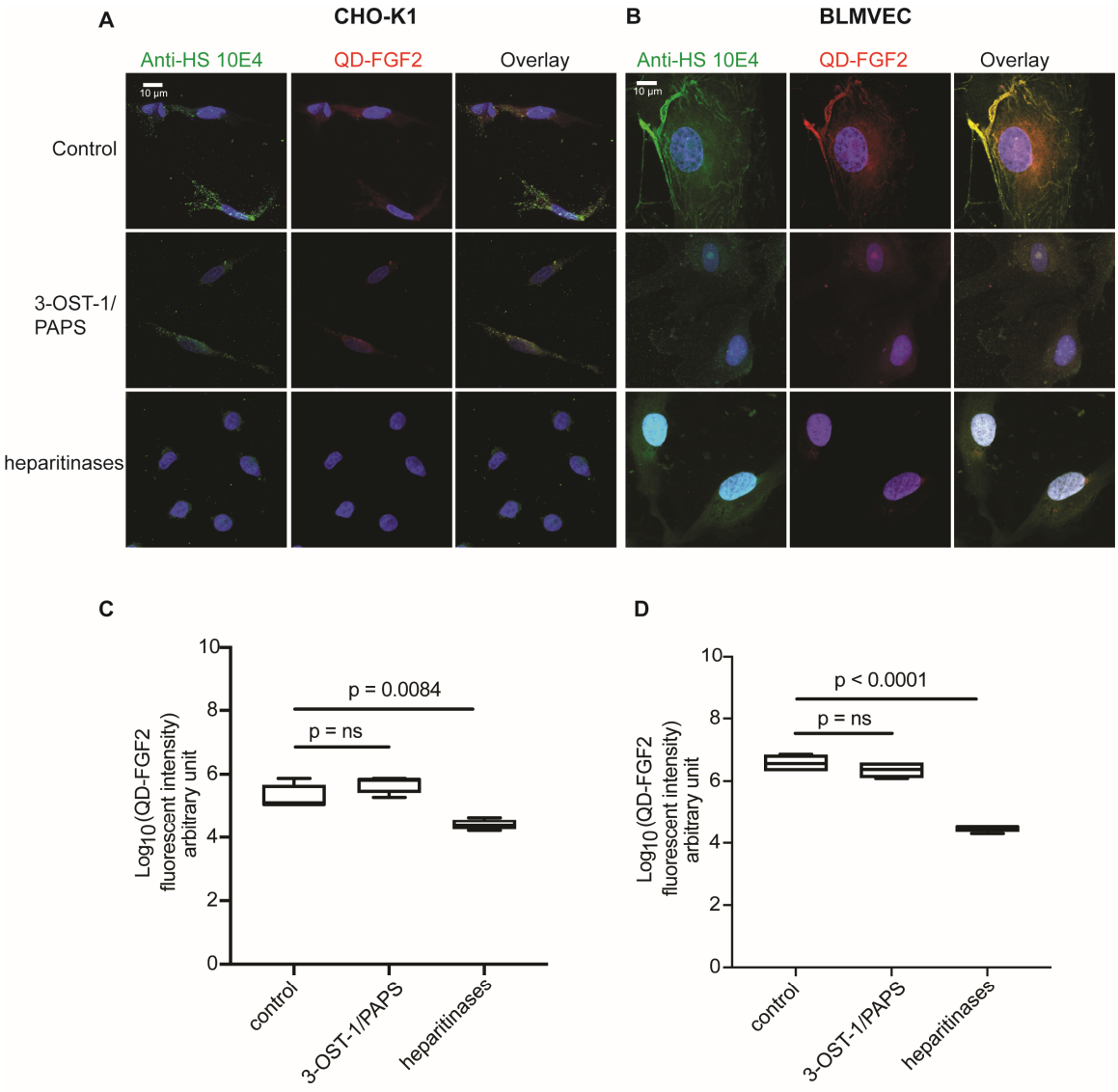

Figure S6. Fluorescence imaging of the common HS tetrasaccharide motif (λ_{EM}= 565 nm for QD-FGF2 (red); λ_{EM}= 519 nm for anti-HS (10E4) antibody (green), and λ_{EM}= 461 nm for DAPI (blue)). (A) CHO-K1 cells lacking 3-OST-1 expression (top); with 3-OST-1 and PAPS added (middle); and with 3-OST-1 and PAPS added plus treated with heparitinases (bottom). Scale bar = 10 µm. (B) Corresponding experiments using wild-type bovine lung microvasculature cells (BLMVECs). (C) and (D) Intensities quantified from Panels A and B, respectively derived from a set of four experiments (N=4). Statistical significance was calculated using student's t test.

13. References

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