Polymeric Fluorescent Heparin as One-Step FRET Substrate of Human Heparanase

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Materials and Methods

Reagents: EDANS and Dabcyl were from AnaSpec (Fremont, CA). HPLC grade acetonitrile and water were procured from Fisher Scientific. All other chemicals, reagents including heparin, sodium salt, and D_2O (NMR) were purchased from Sigma (St. Louis, MO).

Heparin Labelling with EDANS and DABCYL: Synthesis was carried out using carbodiimide chemistry. Briefly, heparin (1 equivalent) was dissolved in dd H_2O to give 0.25 % (w/v) solution. To this, a solution containing 1 or 2 equivalents each DABCYL C2 amine and EDANS in DMSO were added. To this mixture, 20 and 4 equivalents of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxy succinimide (NHS), and the final volume made up to 2 ml with dd H_2O. The mixture was stirred for 20 h at 37 °C and dialyzed extensively against dd H_2O (3500 MWCO), followed by concentration using centricon with 3000 MWCO cutoff membrane. The labeled heparins were purified by G-15 Sephadex size exclusion chromatography (SEC) and characterized using ^{1}H NMR spectroscopy. This reaction was performed multiple times using different batches of commercially available heparin. The results obtained with different batches of heparin-DE were essentially identical.

Another set of labeled heparins were also prepared in which the reducing end of heparin was labeled with either fluoresceinamine-1 or rhodamine 123. Briefly, unfractionated heparin (1 equivalent), 10 equivalents of either fluoresceinamine-1, or rhodamine 123, and 30 equivalents of piconylborane were refluxed in dimethylformamide (DMF) at 60°C for 12 hours. The water soluble product was extracted in a separating funnel from dichloromethane and purified on a G15 Sepharose column. The lyophilized reducing end labeled heparin was then used as a starting material for coupling with other fluorophores (rhodamine 123 or fluoresceinamine-1) using 20 and 4 equivalents of EDC and NHS, respectively.

NMR Calculations for Stoichiometry of Labeling: Stoichiometrices of two FRET labels in the final synthetic heparin product were calculated using ^{1}H NMR spectroscopy (Supplementary Fig. S1). Peaks in unlabeled and FRET-labeled heparins were identified based on literature reports [30]. The peaks corresponding to anomeric protons of heparin were integrated to unity, which represent integral corresponding to the total number of anomeric protons in an average chain of heparin (M_R = 15,000; 50 monosaccharide residues per average chain). Using this as reference integral, the peaks arising from the aromatic protons (i.e., 7.02 – 8.10 ppm for EDANS and 6.98 – 7.95 ppm

for DABCYL C2) of the two FRET labels were integrated. These regions correspond to 7 and 8 protons present in EDANS and DABCYL, respectively. Thus, ratio of integrals of these protons to the anomeric proton integral correspond to the number of labels per average chain of heparin, or alternatively, the stoichiometry of labeling. This stoichiometry is reported in Table 1.

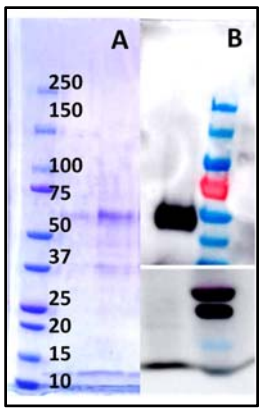
Chromatographic Comparison of Heparin and Heparin-DE Degradation: Size exclusion chromatography (SEC) was used to validate heparin-DE as a substrate of heparanase (Supplementary Fig. S2). The SEC experiments were performed using a Shodex OH Pak SB-802.5 HQ column (8.0 mm × 300 mm; exclusion limit 10,000 Da) on a Shimadzu HPLC system equipped with an RF-10A fluorescence detector. HPSE–heparin-DE reaction mixture (5 µl, 0.5 mg/ml) was injected into the column at RT, eluted with 0.5 ml/min of 30% acetonitrile-water and monitored at 490 nm (λ_{EX} 330 nm).

Cisbio Time-Resolved Fluorescence Assay: HPSE activity assay was performed according to a commercially available kit from Cisbio US (Bedford MA). Briefly, 25 nM heparanase (5 µl) and varying concentrations of suramin dissolved in the heparanase dilution buffer provided with the kit were incubated for 15 min at 37 °C in a 96-well microplate (10 µl total volume). Following pre-incubation, 10 µl of Bio-HS-Eu(K) labeled substrate was added to initiate reaction and incubation for 4 h at 37°C. The reaction was stopped by adding 10 µl of XL665-labeled streptavidin (20 nM final concentration). After 30 min incubation at RT, the signal was measured using a 96-well plate reader (Flexstation 3) at λ_{EM} of 620 and 665 nm (λ_{EX} = 313 nm).

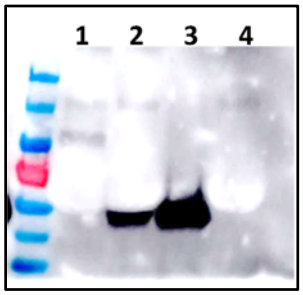
Supplementary Table S1. Assays Reported for Heparanase and their Characteristics.[a,b]

#	Principle	Advantages	Disadvantages	Refs[c]
1	Ultrafiltration and radiolabeling: Measurement of heparanase degradation products	• Most probably sensitive • Rapid processing of samples • Short set up time	• Needs radiolabeled HS (^{35}S) • Limit of detection unknown • Cannot be adapted to a microplate format	13
2	Colorimetry: Heparanase cleavage of biotinylated-HS, covalently bound to a surface, is detected through peroxidase-conjugated streptavidin	• Microplate format enabled • Sensitive • Cell culture adaptable	• Not adaptable for cellular histological imaging • Variable biotinylation could affect the sensitivity • Long time for development of color (3 h)	14
3	Fluorescence: Heparanase cleavage of immobilized HS monitored fluorescence of labeled HS fragments	• HTS enabled • Sensitive • Requires only 20 ng heparanase • Short incubation time (30 – 60 min)	• Not adaptable for cellular histological imaging	15
4	Fluorescence: Heparanase cleavage of immobilized and fluorophore-labeled HSPG quantified using allophycocyanin-labeled streptavidin	• Microplate adaptable • Highly sensitive assay • Detects enzyme as low as 160 ng • Less interference likely at the read λ of 665 nm	• Requires modified HSPG forms as a substrate • Sensitive to different types of HSPGs (high or low molecular weight) • Requires preparation of a special read probe	16
5	Colorimetry: Heparanase cleavage of fondaparinux quantified using a tetrazolium salt	• Highly sensitivity (56 – 224 pM levels) • Microplate adaptable • Less interference at the read λ of 584 nm • A well-defined substrate (one cleavage site)	• Long incubation times of up to 24 h • Color development requires 60 °C for 60 min • Requires a standard curve with *D*-Gal	17
6	UV/Fluorescence: Chromogenic/fluorogenic aryl glucuronides as heparanase substrates	• Homogeneous substrates that show a UV- or fluorescence-signal • Smallest heparanase substrates	• Specific activities appear to be not high (17 to 48 nmol h^{-1} mg^{-1}) (could be a heparanase problem)	18
7	Fluorescence: Heparanase cleavage of fondaparinux quantified using resazurin	• Microplate adaptable • Less interference at the read λ of 590 nm • Substrate cleavage products do not inhibit heparanase	• Overnight incubation at 37 °C necessary • Reduction requires 45 °C for 60 min • Sensitive to resazurin concentration • Confounding results if other reducing sugars present	19
8	Fluorescence: Immobilized biotinylated HS that remains uncleaved by heparanase quantified by streptavidin-conjugated europium	• Microplate adaptable • Cell and tissue extracts adaptable • Optimal at heparanase below 0.5 μg	• Overnight incubation at 37 °C necessary • Requires preparation of two special agents (Immobilizable HS and fluorophore-labeled streptavidin)	19

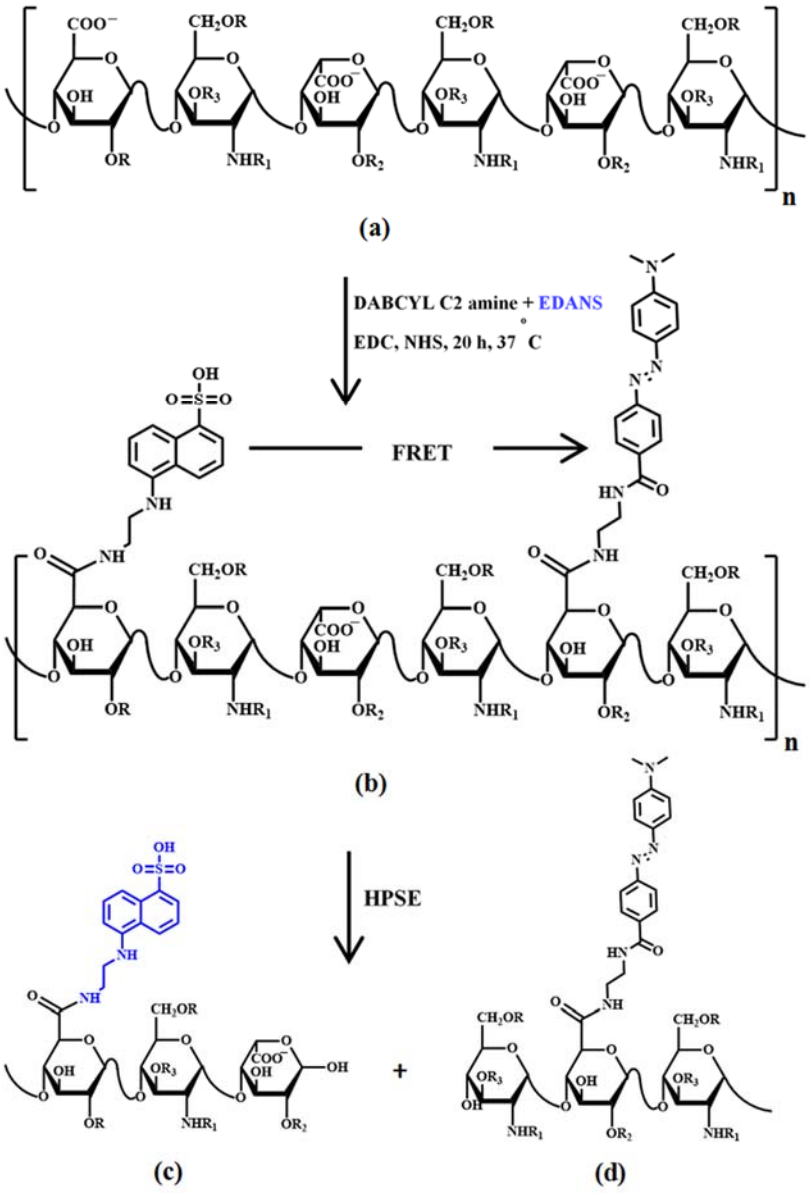
[a] Abbreviations used in the table include: *D*-Gal = *D*-Galactose; *D*-GlcA = *D*-glucuronic acid; HS = heparan sulfate; HSPG = heparan sulfate proteoglycan; HTS = high-throughput screening; [b] In addition to the assays listed in this table, several radiolabeling-based assays have been presented (see refs. 11–13), which suffer from the same limitations of radiolabeling. These are not listed here to keep the listing concise. Further, at least two heparanase kits are commercially available (Cisbio heparanase assay toolbox and Amsbio heparanase assay kit), which are based on references described here including 16 and 32. [c] Only representative references cited here. Note that radio-isotope based detection assays are not included here at all.



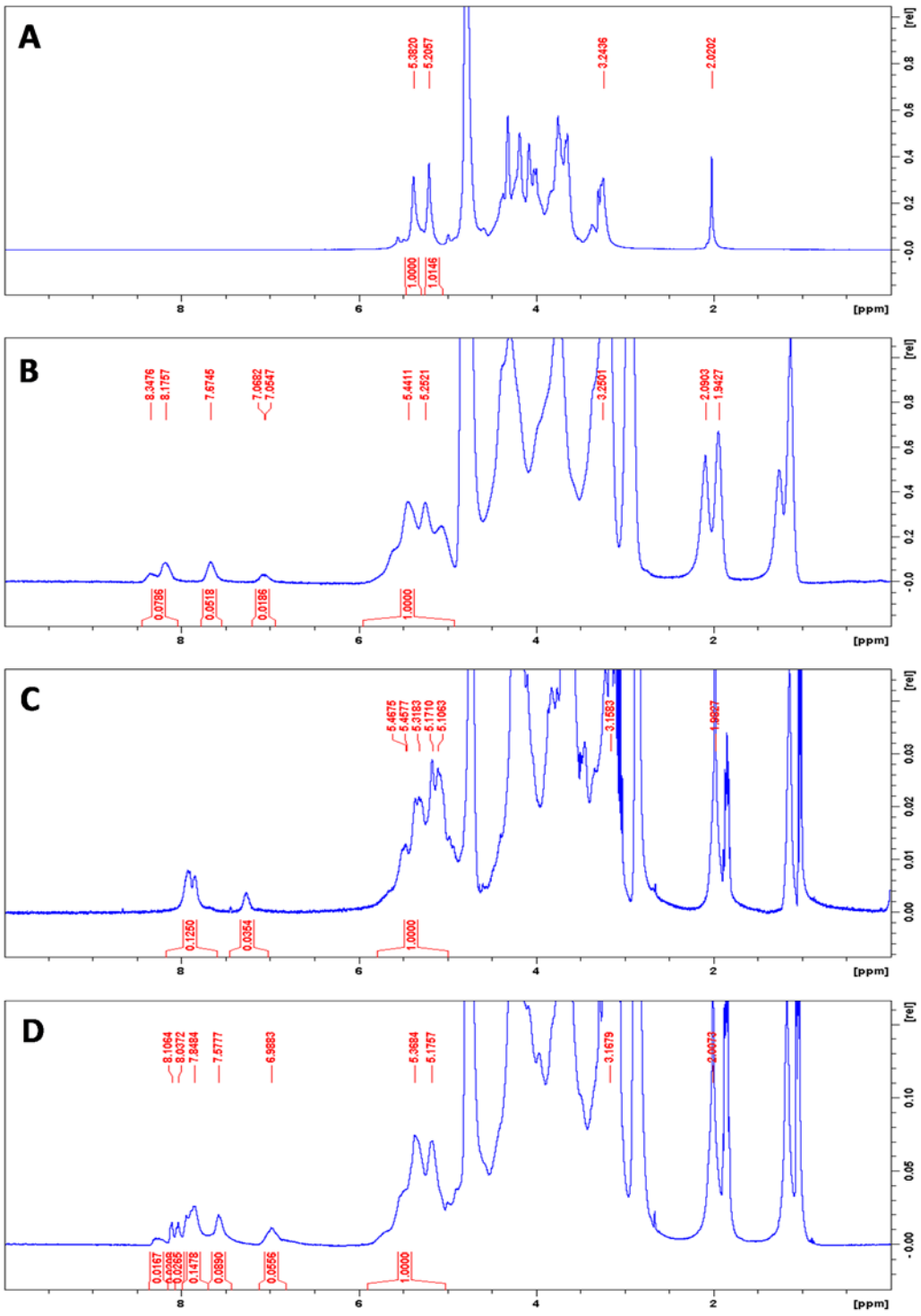
Supplementary Figure S1: A) SDS-PAGE showing large (~50 kDa) and small HPSE subunits. B) Western blot analysis of the large and small subunits. Detection was performed using the corresponding commercially available primary antibodies for the two subunits.



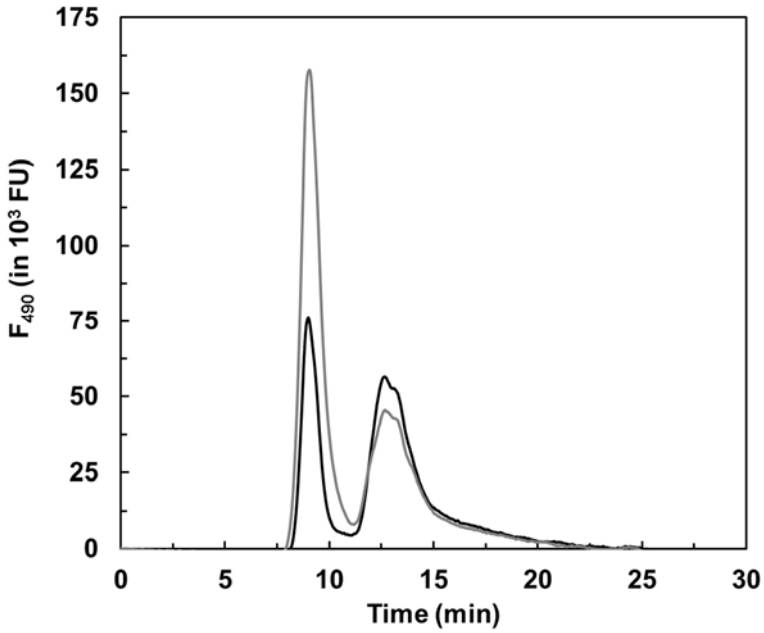
Supplementary Figure S2: Western blot of HPSE expressed under different conditions. Lane 1) Transfected HEK; Lane 2) MCF7 cell line. Lane 3) Insect cells. Lane 4) Cell media. The large subunit was detected using a commercially available primary antibody.



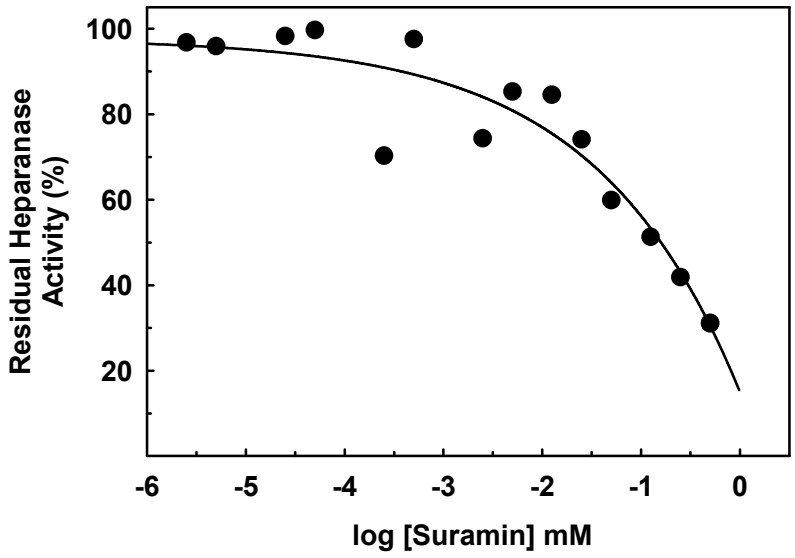
Supplementary Figure S3. Schematic representation of substrate heparin-DE synthesis and overview of the origin of FRET signal. a: heparin; b: heparin-DE; c and d: substrate fragments upon HPSE cleavage. This synthesis was performed with multiple batches of heparin and found to yield essentially identical FRET signal.



Supplementary Figure S4: 400 MHz ^{1}H NMR spectra of heparin and FRET-labeled heparins in D_2O. Peaks at 5.43 and 5.26 ppm indicate the anomeric protons in heparin, whereas peaks in the range of 7 – 9 ppm correspond to EDANS and DABCYL C2. **A)** Unlabeled heparin. **B)** Heparin labeled with 2 equivalents of EDANS. **C)** Heparin labeled with 2 equivalents of DABCYL. **D)** Heparin labeled with 2 equivalents each of EDANS and DABCYL (Heparin DE).



Supplementary Figure S5: SEC–HPLC analysis of heparin–DE in the presence (black) and absence (grey) of heparanase. Heparin–DE (1 mg/ml) was incubated for 4 h at 37 °C with and without with heparanase (1 μM). The digested sample is injected directly on to the Shodex OH Pak SB-802.5 HQ (8.0 mm x 300 mm) size exclusion column. The sample was eluted isocratically using 30% acetonitrile in water and monitored at 490 nm (λex = 330 nm). The heparin peak (~9 min) decreased 2-fold and whereas the fluorescence of the smaller oligosaccharides an increased by 24%.



Supplementary Figure S6: Suramin inhibition of heparanase using a commercial kit from Cisbio (Cisbio US, Bedford, MA). The assay conditions were identical to those of Figure 4. Curve fitting was performed using dose-response relationship. An IC_{50} of 170 ± 9 μM was calculated, which compares favorably with 300 ± 5 μM measured in our FRET assay.