Electronic Supplementary Information

Highly Sensitive and Ratiometric Luminescence Sensing of Heparin through Templated Cyanostilbene Assemblies

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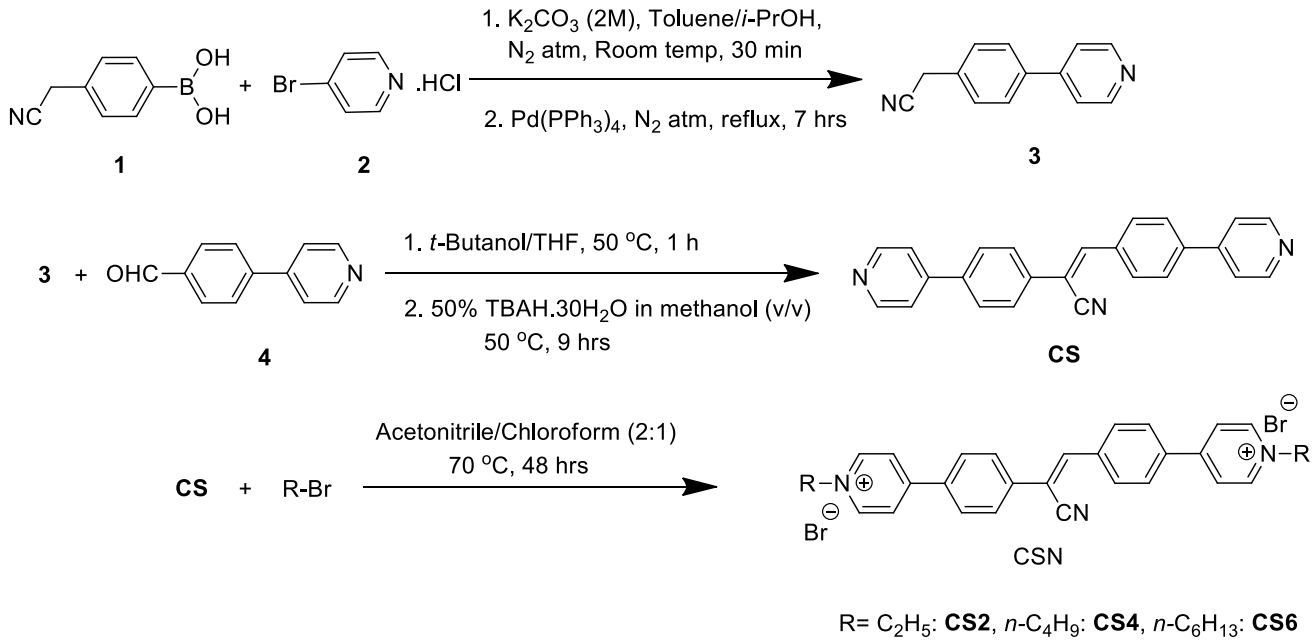
Experimental Procedures

1. Synthetic Schemes and Characterization Data:



Scheme S1- Synthetic route for the preparation of **CSN**s

A. Procedure for synthesis of CS: 2-(4-(pyridin-4-yl)phenyl)acetonitrile (**3**) was first synthesized from (4-(cyanomethyl)phenyl)boronic acid (**1**) and 4-bromopyridine hydrochloride (**2**) according to a literature procedure.[1] Then, (Z)-2,3-bis(4-(pyridin-4-yl)phenyl)acrylonitrile (**CS**) was prepared by a slight modification of the reported procedure.[S1] 2-(4-(pyridin-4-yl)phenyl)acetonitrile (**3**) (360 mg, 1.86 mmol) and 4-(pyridin-4-yl)benzaldehyde (**4**) (340 mg, 1.86 mmol) were taken in a 10 mL round bottom flask and dissolved in 6 mL *tert*-butanol and 0.30 mL tetrahydrofuran mixture. The reaction mixture was stirred for 1 hour at 50 °C. 0.8 mL of 50% tetrabutylammonium hydroxide.30 H_2O (TBAH.30H_2O) in methanol (V/V) was then added dropwise over a period of 15 min at 50 °C until the reaction mixture assumed a maroon color. Stirring was continued for 9 more hours at 50 °C and then the reaction mixture was cooled to room temperature. The pale yellowish white precipitate formed was then filtered and washed with *tert*-butanol for 3-4 times and finally dried under vacuum (340 mg, 0.93 mmol). Yield = 50%. **^{1}H NMR (400 MHz, $CDCl_3$):** δ (ppm) = 8.71 (m, 4H), 8.05 (d, J = 8 Hz, 2H), 7.84 (d, J = 8 Hz, 2H), 7.76 (t, J = 8 Hz, 4H), 7.66 (s, 1H), 7.56 (m, 4H).

B. General procedure for the synthesis of CSNs (N = 2, 4, 6): CS (55 mg, 1.5 mmol) and corresponding alkyl halide (30 mmol) were taken in a 10 mL reaction tube and dissolved in 2 mL acetonitrile and 1 mL chloroform solvent mixture. The reaction mixture was stirred at 70 °C under sealed condition for 48 hours

and then allowed to cool at room temperature. The yellow precipitate formed was filtered and washed with cold chloroform for 3-4 times and finally dried under vacuum.

CS2: From 55 mg of **CS**, 85 mg (0.147 mmol) of **CS2** was obtained as a bright yellow powder. Yield = 98%.

^{1}H NMR (400 MHz, DMSO-D$_6$): δ(ppm) = 9.20 (t, J = 8 Hz, 4H), 8.63 (d, J = 8 Hz, 4H), 8.46 (s, 1H), 8.31 (t, J = 8 Hz, 4H), 8.24 (d, J = 8 Hz, 2H), 8.09 (d, J = 8 Hz, 2H), 4.66 (q, J= 8 Hz, 4H), 1.59 (t, J = 8 Hz, 6H).

^{13}C NMR (125 MHz, DMSO-D$_6$): δ(ppm) = 153.30, 153.27, 144.75, 144.70, 143.25, 136.69, 130.33, 129, 128.73, 127.06, 124.67, 124.58, 117.30, 111.23, 55.57, 16.29.

HRMS (ESI): m/z calculated for $C_{29}H_{27}N_3^{2+}$: 208.6097; found: 208.6097.

CS4: From 55 mg of **CS**, 88 mg (0.14 mmol) of **CS4** was obtained as a bright yellow powder. Yield = 93%.

^{1}H NMR (400 MHz, DMSO-D$_6$): δ(ppm) = 9.19 (t, J = 8 Hz, 4H), 8.63 (d, J = 8 Hz, 4H), 8.45 (s, 1H), 8.31 (t, J = 8 Hz, 4H), 8.24 (d, J = 8 Hz, 2H), 8.09 (d, J = 8 Hz, 2H), 4.63 (q, J= 8 Hz, 4H), 1.95 (m, 4H), 1.34 (m, 4H), 0.95 (t, J = 8 Hz, 6H).

^{13}C NMR (125 MHz, DMSO-D$_6$): δ(ppm) = 153.36, 153.33, 144.93, 144.88, 143.27, 136.71, 130.34, 129.03, 128.76, 127.06, 124.69, 124.59, 117.31, 111.26, 59.82, 32.61, 18.78, 13.33.

HRMS (ESI): m/z calculated for $C_{33}H_{35}N_3^{2+}$: 236.6410; found: 236.6410.

CS6: From 55 mg of **CS**, 90 mg (0.13 mmol) of **CS6** was obtained as a bright yellow powder. Yield = 87%.

^{1}H NMR (400 MHz, DMSO-D$_6$): δ(ppm) = 9.20 (t, J = 8 Hz, 4H), 8.64 (d, J = 8 Hz, 4H), 8.46 (s, 1H), 8.32 (t, J = 8 Hz, 4H), 8.24 (d, J = 8 Hz, 2H), 8.09 (d, J = 8 Hz, 2H), 4.62 (q, J= 8 Hz, 4H), 1.96 (m, 4H), 1.31 (br s, 12H), 0.88 (t, J = 8 Hz, 6H)

^{13}C NMR (125 MHz, DMSO-D$_6$): δ(ppm) = 153.34, 153.31, 144.92, 144.87, 143.27, 136.73, 130.34, 129.03, 128.76, 127.06, 124.68, 124.58, 117.31, 111.26, 60.04, 59.99, 30.63, 30.57, 25.08, 21.85, 13.81.

HRMS (ESI): m/z calculated for $C_{37}H_{43}N_3^{2+}$: 264.6723; found: 264.6729.

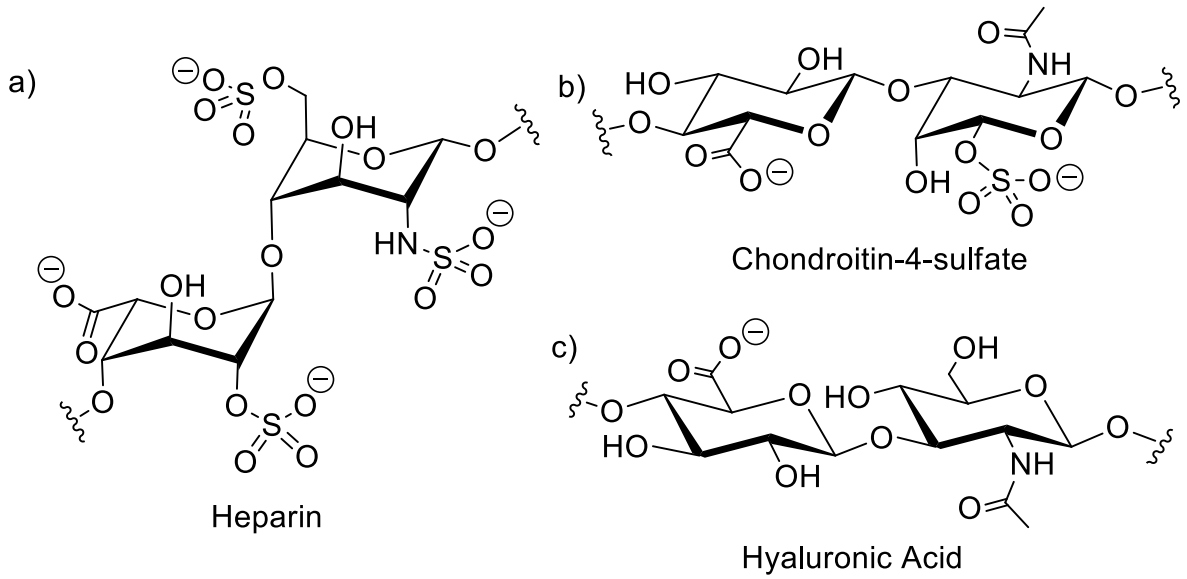


Fig. S1- Common repeat units of heparin, chondroitin-4-sulfate and hyaluronic acid.

2. Preparation of Solutions:

A. Preparation of solution of CSNs:

Initially, stock solutions of **CSN**s (N = 2, 4, 6) were prepared by dissolving the solid powders in spectroscopic grade dimethyl sulfoxide (DMSO). These concentrated DMSO solutions were then diluted to 5 mM tris-HCl buffer made on Milli-Q water to get desired solutions having 1% DMSO as the final DMSO fraction. Solutions of **CS2**, **CS4** and **CS6** in this buffered medium were equilibrated for 10 mins before each titration.

B. Preparation of heparin solution:

The disaccharide unit shown in Fig. S1a is taken as the repeat unit of heparin for the molecular weight calculation. Though the supplied heparin contains only 30-40% materials with the active sequence of repeat units, the whole sample can still bind through the anionic polysaccharide unit irrespective of whether the repeat units are in active sequence or not. The molecular weight of the repeat unit is 665.40 g/mole. Heparin stock solutions were prepared in buffer and further diluted during titration.

C. Preparation of solution of other analytes:

Stock solutions of chondroitin-4-sulfate (**ChS**) (Fig. S1b) and hyaluronic acid (**HA**) (Fig. S1c) were prepared as 1.33 mg/mL in working buffer (2.7 mM for **ChS** and 3.3 mM for **HA**) and further diluted accordingly during titration. Stock solution of protamine sulfate (**PS**) was prepared in buffer using its molecular weight as 4500 Daltons and further diluted during titration.

D. Extraction of human serum:

Blood samples were collected from a group of healthy males of age group 22-30 and then kept at room temperature for 1 hr. The samples were then centrifuged at 1500 rpm for 15 min at room temperature.

The supernatant was collected and again centrifuged at 2500 rpm for 15 min at room temperature. Finally, the serum was collected and incubated at 4 °C overnight and stored at -20 °C.

E. Extraction of human plasma:

Blood samples from a group of healthy males (age group 22-30) were collected in collection tubes containing 3.8% sodium citrate solution (9:1 v/v blood:sodium citrate). The citrated samples were then centrifuged for 15 mins at 2500 rpm at 4 °C. The supernatant (platelet-free citrated plasma) was collected and stored at -20 °C.

3. Titration Procedures:

During the fluorometric titration, the addition of any of the analytes (heparin, chondroitin-4-sulfate, hyaluronic acid or protamine sulfate) was every time performed to a freshly equilibrated buffered solution of **CSN**s to avoid any photoreaction of the cyanostilbene unit. After each addition of any analyte, 10 min equilibration time was given and then the spectrum was recorded.

Titration in buffer containing 50% human serum: To a 1.98 mL solution of sensor (**CS6**, 50.50 µM) in buffer containing 50% human serum, 20 µL of heparin stocks in 100% human serum was added during each titration so that the final concentration of sensor (**CS6**) became 50 µM.

Titration in buffer containing 50% human plasma: To a 1.98 mL solution of sensor (**CS6**, 50.50 µM) in buffer containing 50% human plasma, 20 µL of heparin stocks in 100% human plasma was added during each titration so that the final concentration of sensor (**CS6**) became 50 µM.

Titration in buffer containing 60% human plasma: To a 1.98 mL solution of sensor (**CS6**, 60.60 µM) in buffer containing 60% human plasma, 20 µL of heparin stocks in 100% human plasma was added during each titration so that the final concentration of sensor (**CS6**) became 60 µM.

Titration in buffer containing 50% FBS: To a 1.98 mL solution of sensor (**CS6**, 50.50 µM) in buffer containing 50% FBS, 20 µL of heparin stocks in 100% FBS was added during each titration so that the final concentration of sensor (**CS6**) became 50 µM.

4. Limit of Detection (LOD) Calculation:

Limit of detection was calculated using the equation 1.

Limit of detection (LOD) = $3\sigma/m$ …. (equation 1)[2]

where, σ is the standard deviation of the intensity of free **CSN** (at 536 nm in buffer) or intensity ratio (I_{541}/I_{456} in serum and plasma samples) (determined based on five measurements), and m is the slope of

the intensity (at 536 nm in buffer) or intensity ratio (I_{541}/I_{456} in serum and plasma samples) vs the concentration of heparin plot.

5. Quantum Yield Calculation Method:

A. Quantum yield measurement of monomeric solutions in buffer:

The quantum yields of the monomeric solutions of **CS2**, **CS4** and **CS6** in buffer were measured by

$$Q_f = Q_{ref} \times \frac{a_{ref}}{a_{sam}} \times \frac{A_{sam}}{A_{ref}} \times \left[\frac{\eta_{sam}}{\eta_{ref}}\right]^2$$

employing the reference method using the following equation.[3]

Where Q_f and Q_{ref} are the quantum yields (QY), A_{sam} and A_{ref} are the areas under the fluorescence spectrum, a_{sam} and a_{ref} are the absorbance at excitation wavelength (λ_{ex} = 366 nm) of the sample and the reference, respectively. η_{sam} and η_{ref} are the refractive indices of the solvents where the sample and the reference were dissolved, respectively. Quinine sulfate in 0.1 M H_2SO_4 was used as the reference dye.

B. Absolute quantum yield measurement of in presence of heparin:

Absolute quantum yields of **CS2** (5 µM), **CS4** (5 µM) and **CS6** (5 µM) in the presence of heparin were measured using integrating sphere.

6. Activated Partial Thromboplastin Time (aPTT) Test Procedure:

50 mg of silica (100-200 mesh) and mono-n-dodecyl phosphate (0.4mg/mL stock, incubated at 37 ^oC for at least 2 min) were taken in a 2 mL glass vial. 0.1 mL of plasma or heparin containing plasma (incubated at 37 ^oC for 2 min) was added to it and after a gentle stirring, the vial was kept at 37 ^oC for exactly 5 min. Then aqueous $CaCl_2$ solution (0.025 M, incubated at 37 °C for at least 2 min) was added to it and the vial was kept at 37 °C for 20 sec. Then in every 10 sec, clot formation was checked by gently tilting the vial. The clot formation time was noted down.

The aforementioned procedure was employed for the lab heparin and a standard heparin injection solution. Subsequently, aPTT ratios were plotted against concentration of the lab heparin or the activity of the standard heparin. From the calibration curve of the standard heparin, activity (U/mL) of our lab heparin at different concentrations was calculated.

Results and Discussion

1. Spectroscopic Studies of CSNs in Aqueous Buffer:

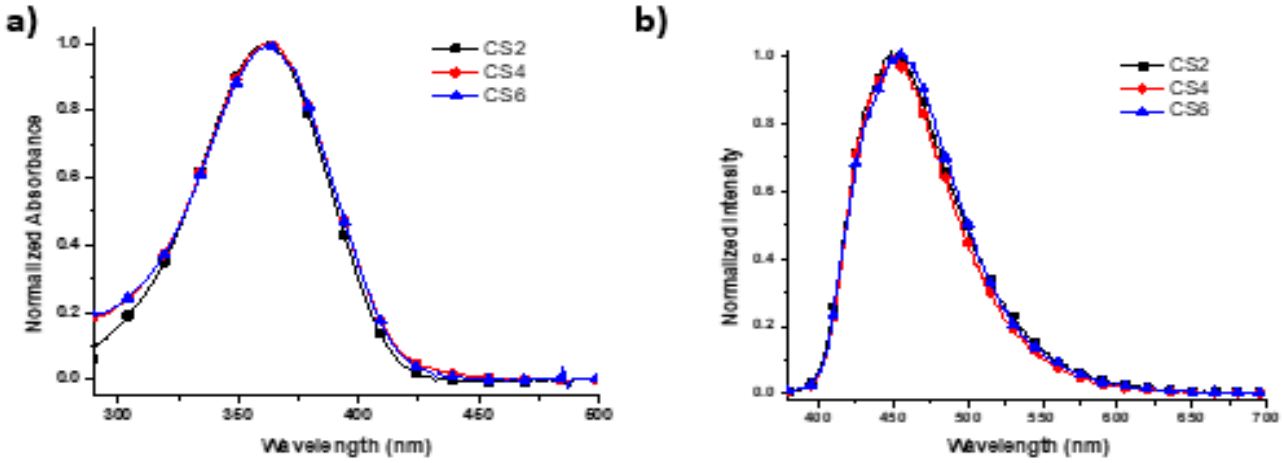


Fig. S2- Normalized (a) absorption and (b) emission spectra of **CS2**, **CS4** and **CS6** in aqueous buffer (5 mM tris-HCl, 99:1 water/DMSO, pH 7.4).

2. Heparin Sensing in Buffer:

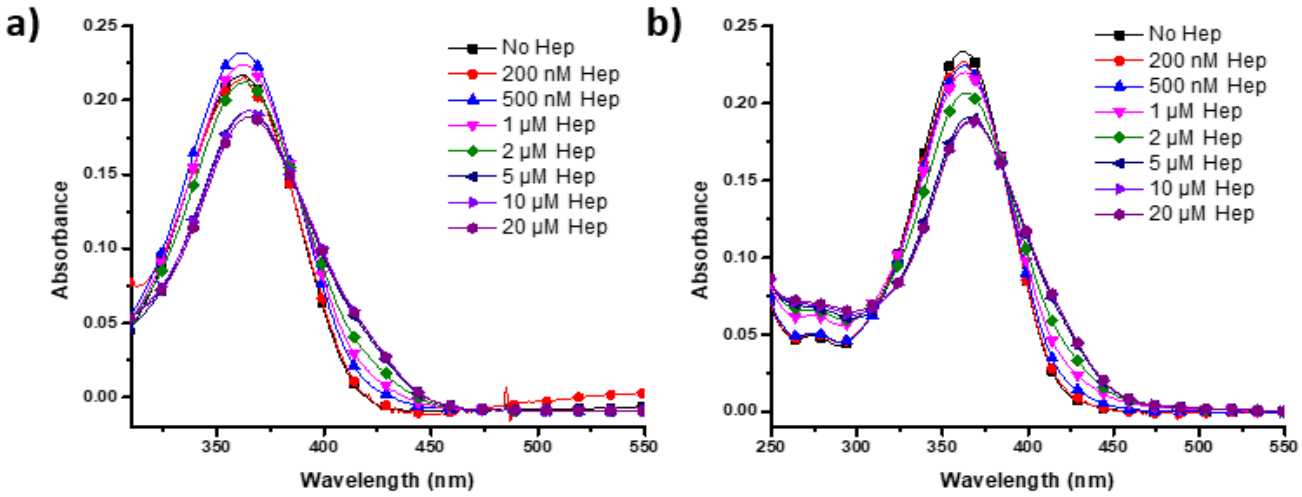


Fig. S3- Absorption spectral changes of (a) **CS2** (5 µM) and (b) **CS4** (5 µM) upon addition of heparin in aqueous buffer (5 mM tris-HCl, 99:1 water/DMSO, pH 7.4).

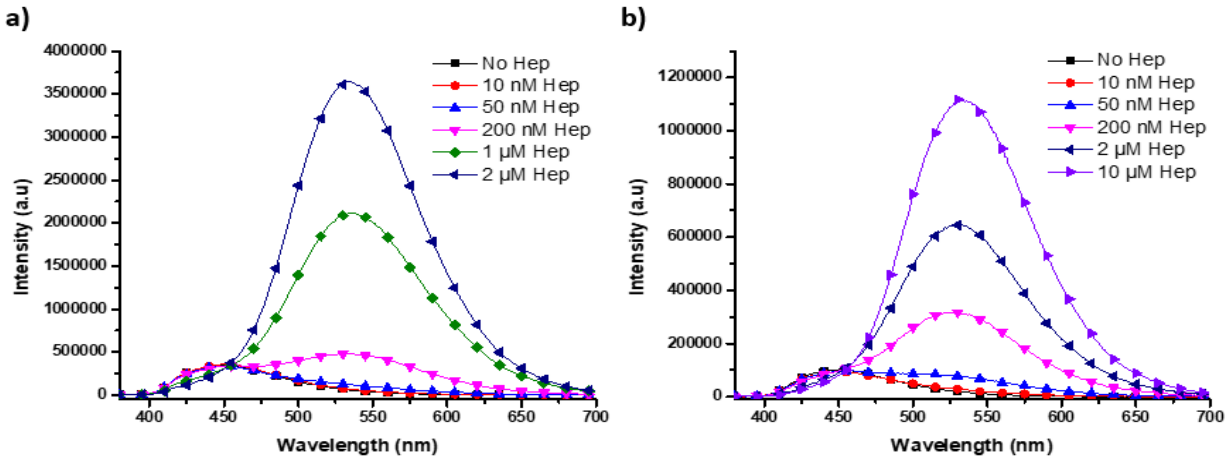


Fig. S4- Emission spectral changes of (a) **CS2** (5 µM) and (b) **CS4** (1.2 µM) upon addition of heparin in aqueous buffer (5 mM tris-HCl, 99:1 water/DMSO, pH 7.4).

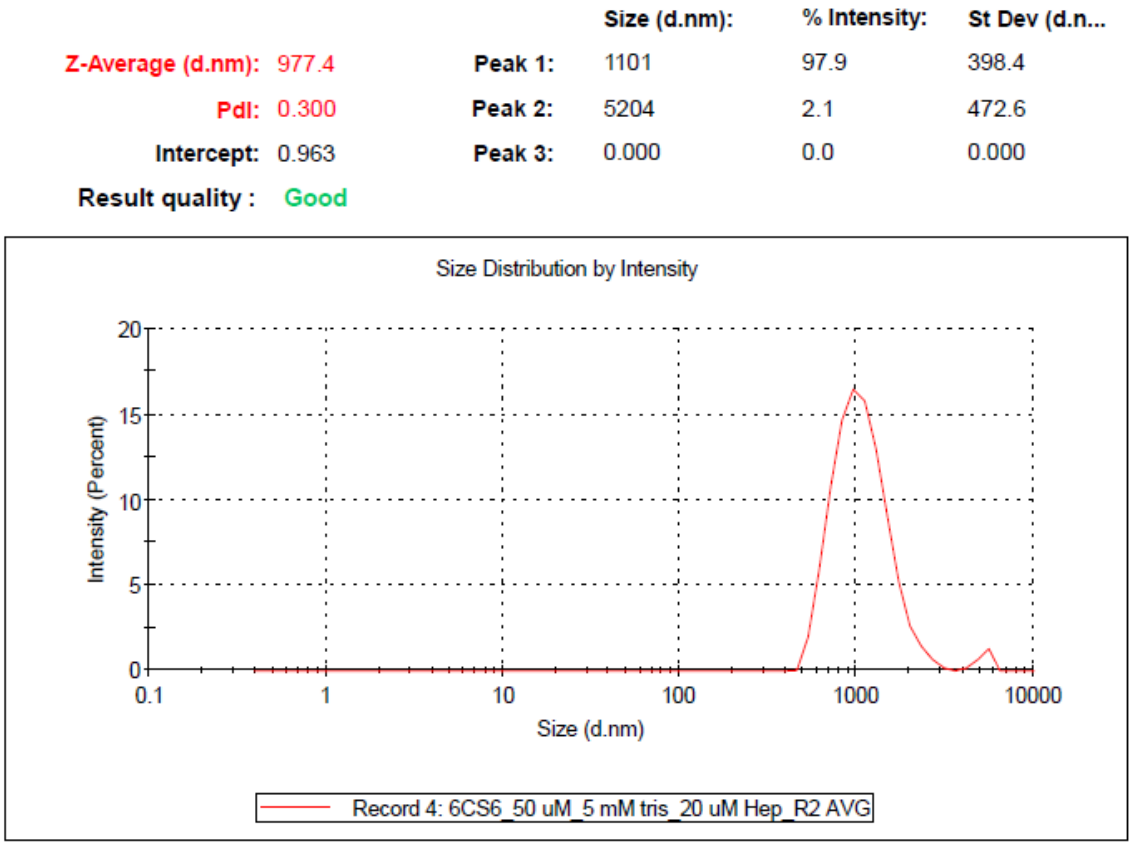


Fig. S5- Size distribution of **CS6** (50 µM) upon addition of 20 µM of heparin in aqueous buffer.

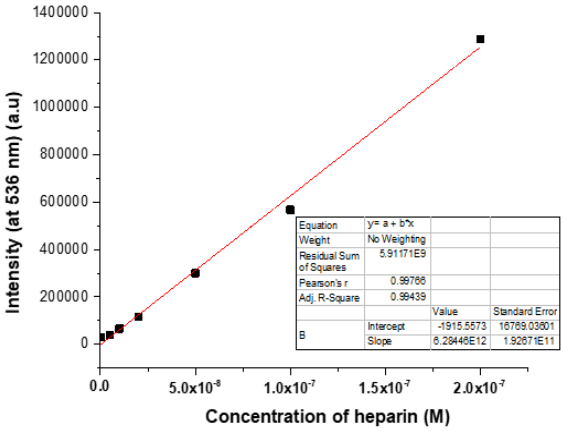


Fig. S6- Linear fit of I_{536} vs heparin concentration for **CS6** (1.2 µM) in aqueous buffer.

Limit of Detection (LOD) Calculation in aqueous buffer: Standard deviation (σ) at 536 nm without heparin = 285.766.

Slope of the linear fit (m) = 6.28446×10^{12}

Limit of Detection (LOD) = 140 pM (93 pg/mL)

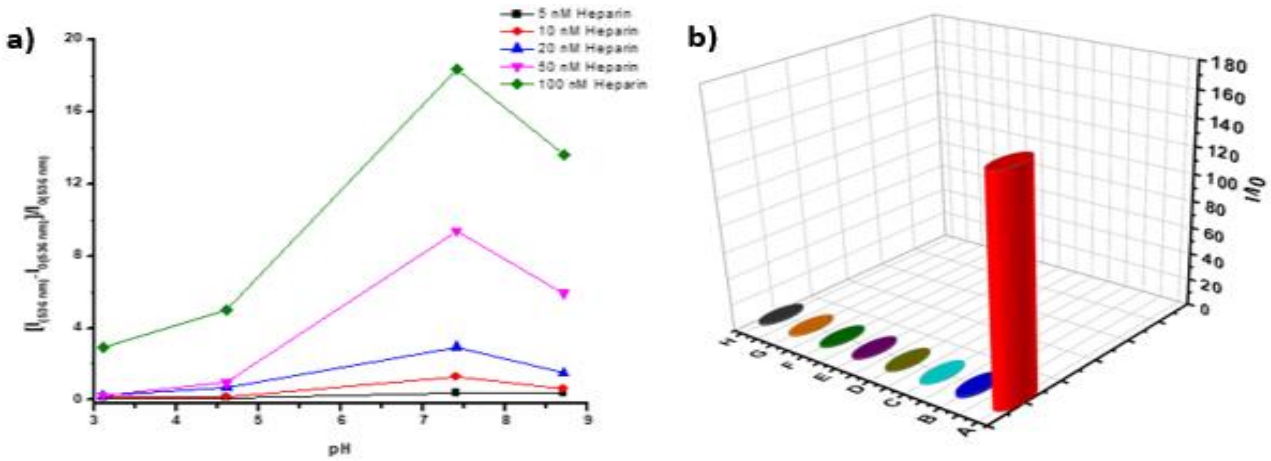


Fig. S7- (a) Heparin binding by **CS6** (1.2 µM) in aqueous buffer having different pH. (b) Comparison bar diagram of I/I_0 (at 536 nm) of **CS6** (1.2 µM) in aqueous buffer upon addition of 2 µM of each of heparin (A), ATP (B), PPi (C), HPO_4^{2-} (D), $H_2PO_4^-$ (E), SO_4^{2-} (F), CO_3^{2-} (G), CH_3COO^- (H).

3. Heparin Sensing in Buffer Containing 150 mM NaCl:

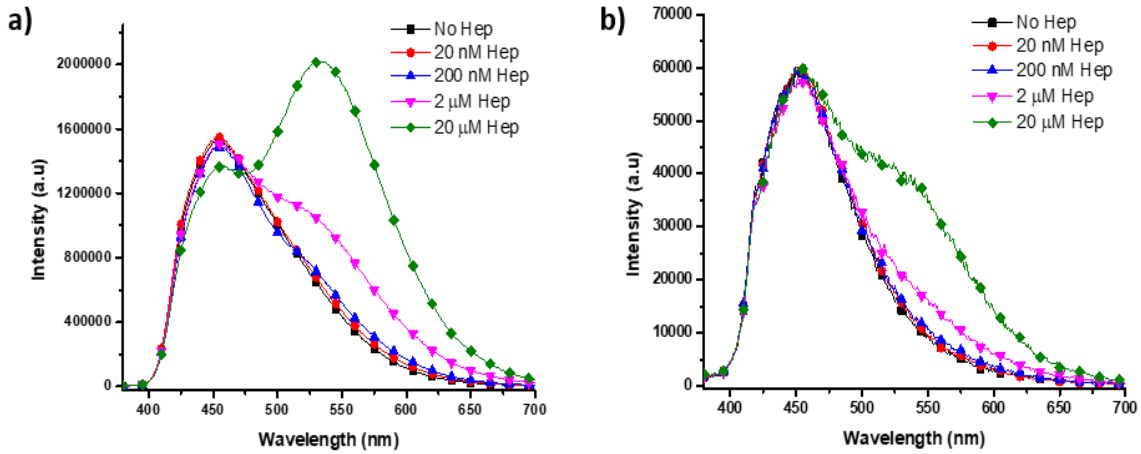


Fig. S8- Emission spectral changes of (a) **CS2** (50 μM) and (b) **CS6** (3 μM) upon addition of heparin in aqueous buffer (5 mM tris-HCl, 99:1 water/DMSO, pH 7.4) containing 150 mM NaCl.

4. Heparin Sensing in Human Plasma:

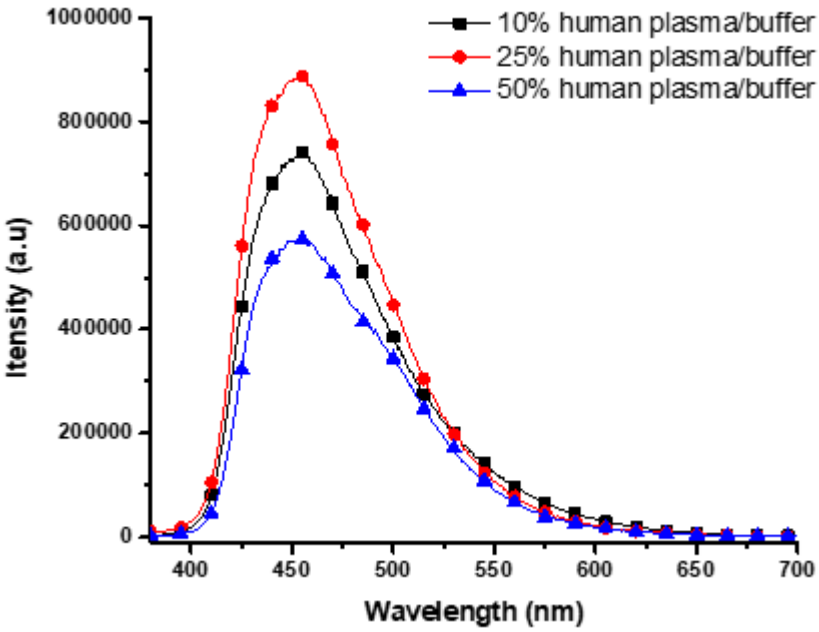


Fig. S9- Emission spectral changes of **CS6** (50 μM) in aqueous buffer containing different contents of human plasma.

Heparin Sensing in 2% Human Plasma:

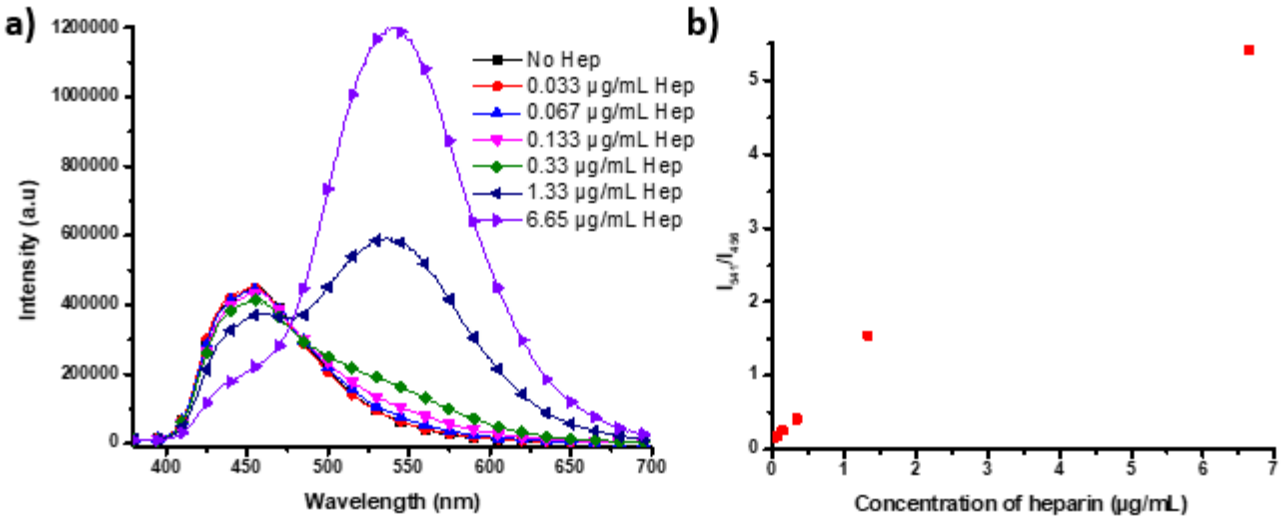


Fig. S10- (a) Emission spectral changes of **CS6** (5 µM, λ_{ex} = 365 nm) upon addition of heparin in aqueous buffer containing 2% human plasma. (b) I_{541}/I_{456} vs. heparin concentration for (a).

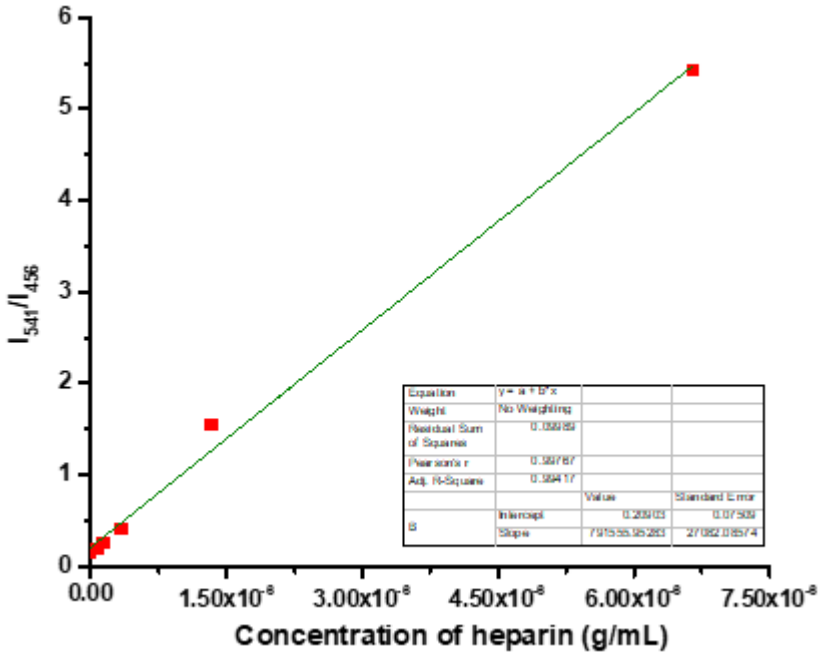


Fig. S11- Linear fit of I_{541}/I_{456} vs heparin concentration for **CS6** (5 µM) in buffer containing 2% human plasma.

Limit of Detection (LOD) calculation in buffer containing 2% human plasma:

Standard deviation (σ) for I_{541}/I_{456} without heparin = 0.00867

Slope of the linear fit (m) = 791555.953

Limit of Detection (LOD) = 0.033 µg/mL (49 nM)

Heparin Sensing in 5% Human Plasma:

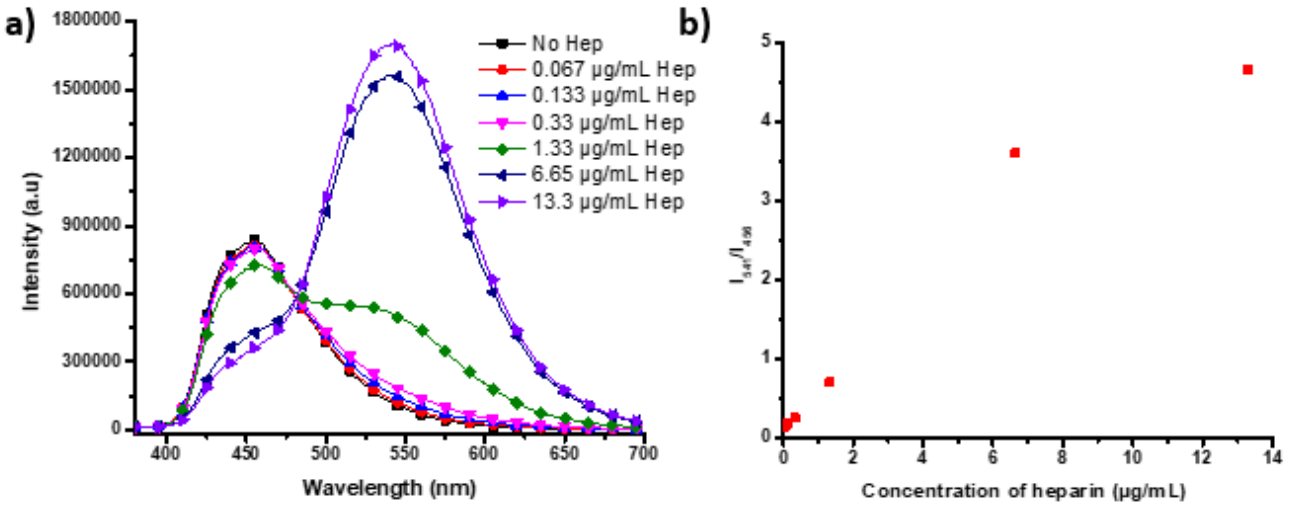


Fig. S12- (a) Emission spectral changes of **CS6** (10 µM, λ_{ex} = 365 nm) upon addition of heparin in aqueous buffer containing 5% human plasma. (b) I_{541}/I_{456} vs. heparin concentration for (a).

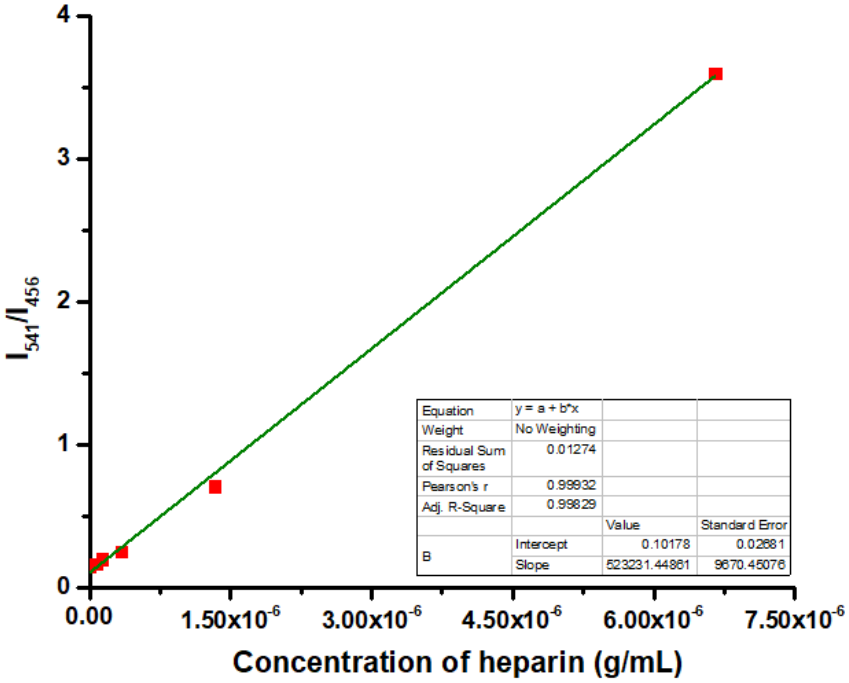


Fig. S13- Linear fit of I_{541}/I_{456} vs heparin concentration for **CS6** (10 µM) in buffer containing 5% human plasma.

Limit of Detection (LOD) calculation in buffer containing 5% human plasma:

Standard deviation (σ) for I_{541}/I_{456} without heparin = 0.00924

Slope of the linear fit (m) = 523231.45

Limit of Detection (LOD) = 0.053 µg/mL (80 nM)

Heparin Sensing in 10% Human Plasma:

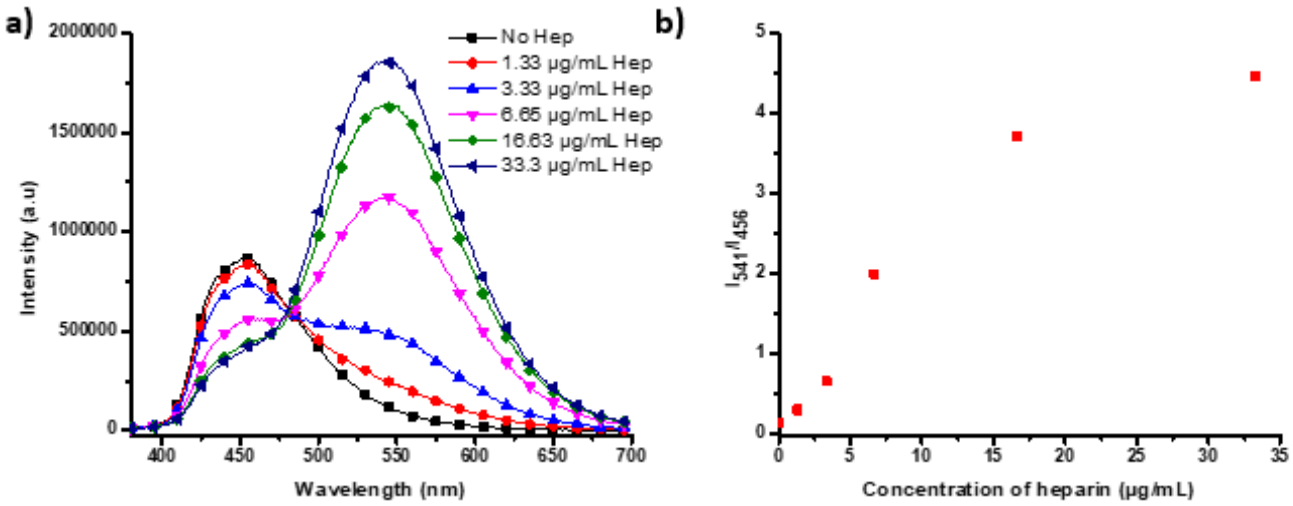


Fig. S14- (a) Emission spectral changes of **CS6** (20 µM, λ_{ex} = 365 nm) upon addition of heparin in aqueous buffer containing 10% human plasma. (b) I_{541}/I_{456} vs. heparin concentration for (a).

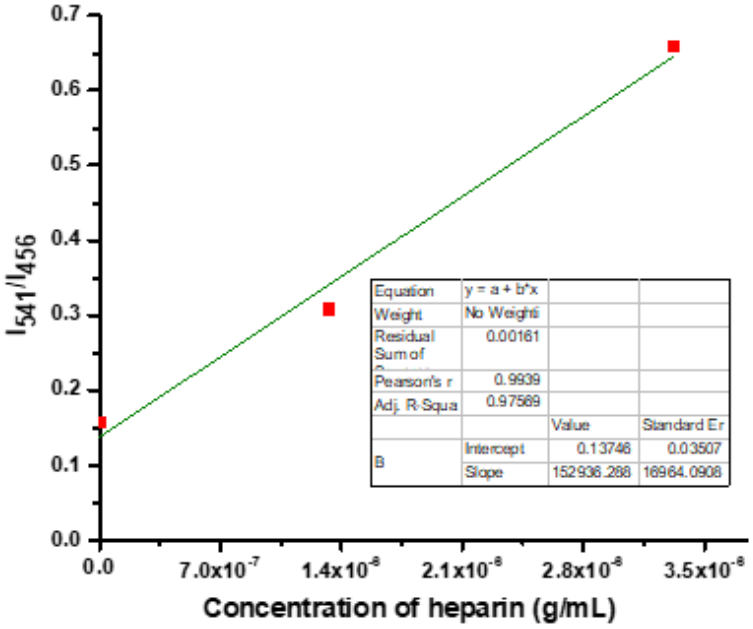


Fig. S15- Linear fit of I_{541}/I_{456} vs heparin concentration for **CS6** (20 µM) in buffer containing 10% human plasma.

Limit of Detection (LOD) calculation in buffer containing 10% human plasma:

Standard deviation (σ) for I_{541}/I_{456} without heparin = 0.0074

Slope of the linear fit (m) = 152936.288

Limit of Detection (LOD) = 0.145 µg/mL (218 nM)

Heparin Sensing in 25% Human Plasma:

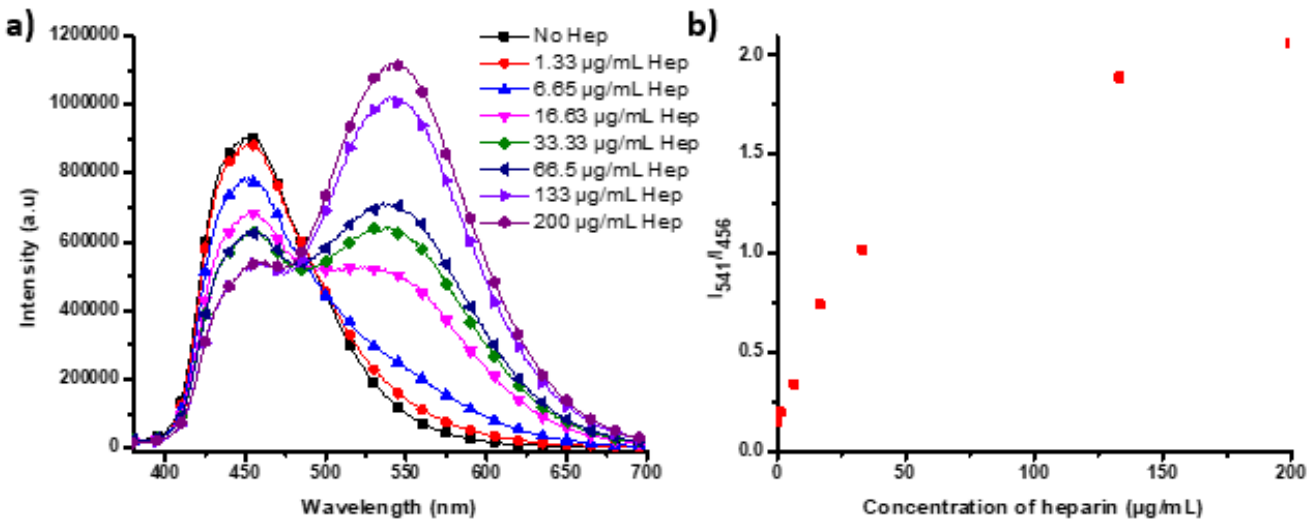


Fig. S16- (a) Emission spectral changes of **CS6** (35 µM, λ_{ex} = 365 nm) upon addition of heparin in aqueous buffer containing 25% human plasma. (b) I_{541}/I_{456} vs. heparin concentration for (a).

Heparin Sensing in 50% Human Plasma:

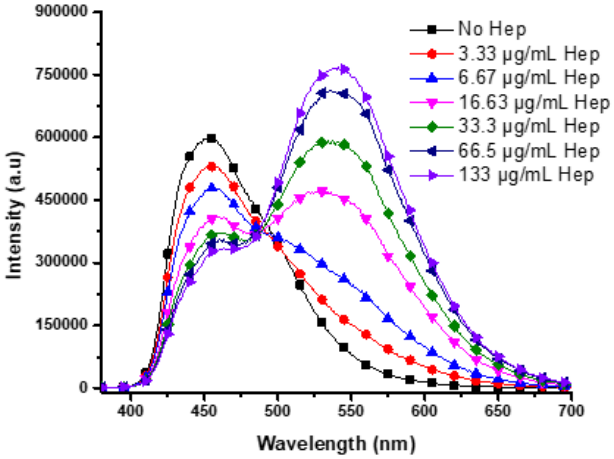


Fig. S17- Emission spectral changes of **CS6** (50 µM) upon addition of lab heparin (delivered in 100% human plasma) in 50% human plasma in buffer.

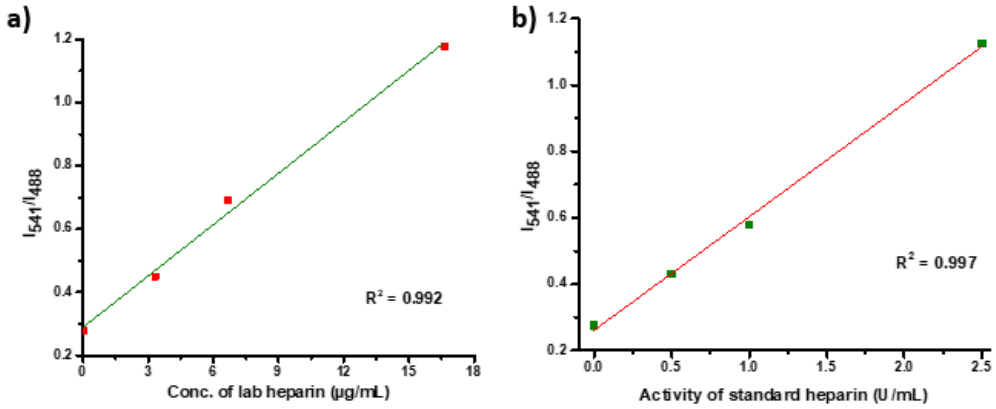


Fig. S18- (a) I_{541}/I_{488} vs. concentration of lab heparin and (b) I_{541}/I_{488} vs. activity of heparin injection in 50% human plasma using **CS6** (50 µM).

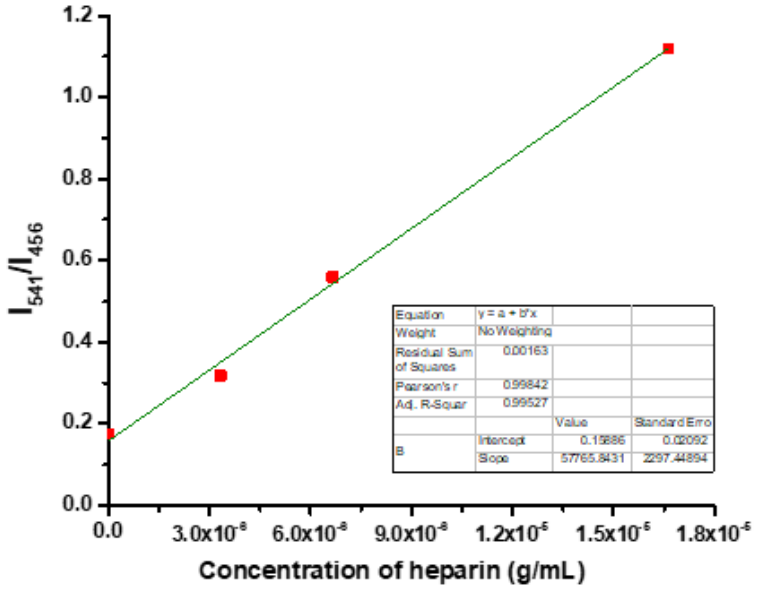


Fig. S19- Linear fit of I_{541}/I_{456} vs heparin concentration for **CS6** (50 µM) in buffer containing 50% human plasma.

Limit of Detection (LOD) Calculation in buffer containing 50% human plasma: Standard deviation (σ) for I_{541}/I_{456} without heparin = 0.0133

Slope of the linear fit (m) = 57765.843

Limit of Detection (LOD) = 0.69 µg/mL (1.04 µM)

Heparin Sensing in 60% Human Plasma:

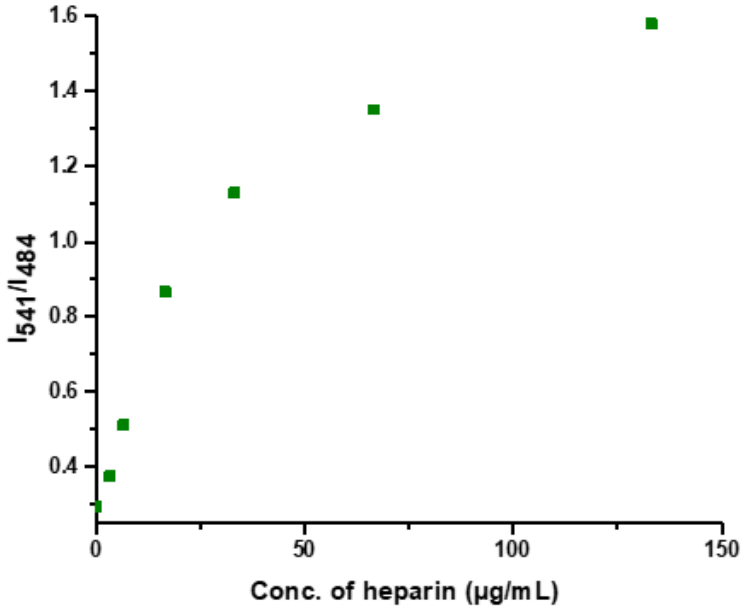


Fig. S20- I_{541}/I_{484} vs heparin concentration for **CS6** (60 µM) in buffer containing 60% human plasma.

5. Heparin Sensing in Serum:

Heparin Sensing in Buffer Containing 50% Human Serum:

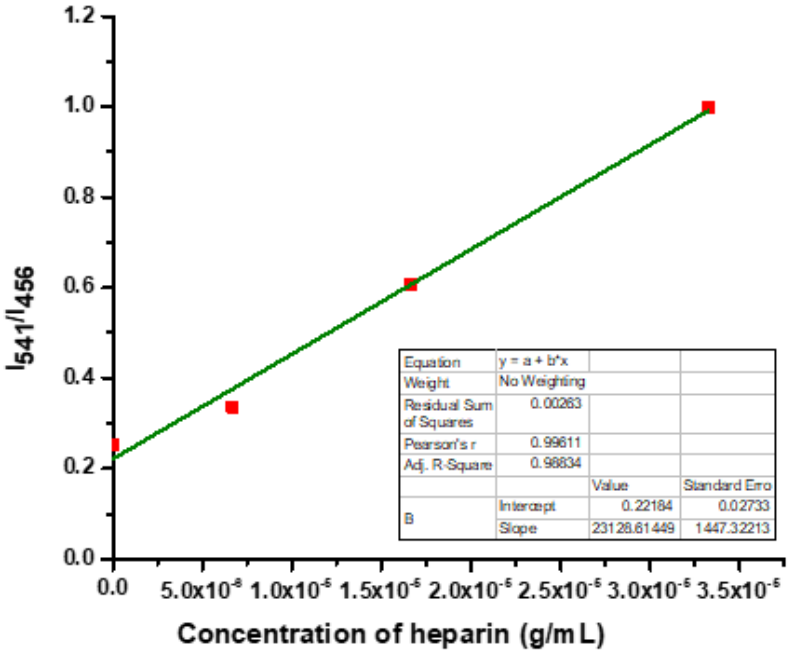


Fig. S21- Linear fit of I_{541}/I_{456} vs heparin concentration for **CS6** (50 µM) in buffer containing 50% human serum.

Limit of Detection (LOD) Calculation in buffer containing 50% human serum: Standard deviation (σ) for I_{541}/I_{456} without heparin = 0.0192

Slope of the linear fit (m) = 23126.6145

Limit of Detection (LOD) = 2.49 µg/mL (3.74 µM)

Heparin Sensing in Buffer Containing 50% Fetal Bovine Serum (FBS):

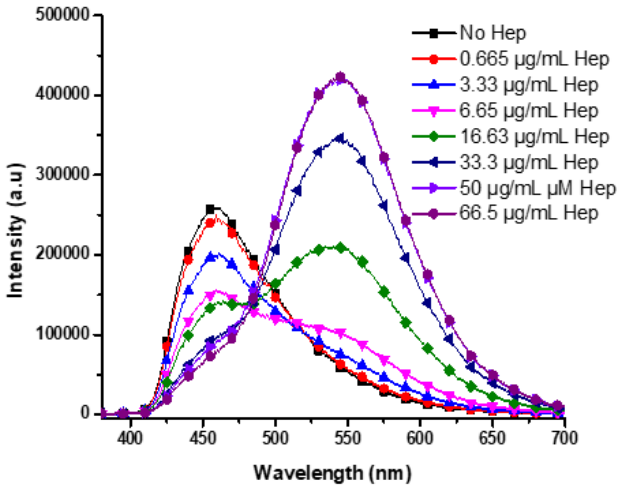


Fig. S22- Emission spectral changes of **CS6** (50 µM, λ_{ex} = 365 nm) upon addition of heparin in aqueous buffer containing 50% fetal bovine serum (**FBS**).

6. Heparin binding in presence of bovine serum albumin:

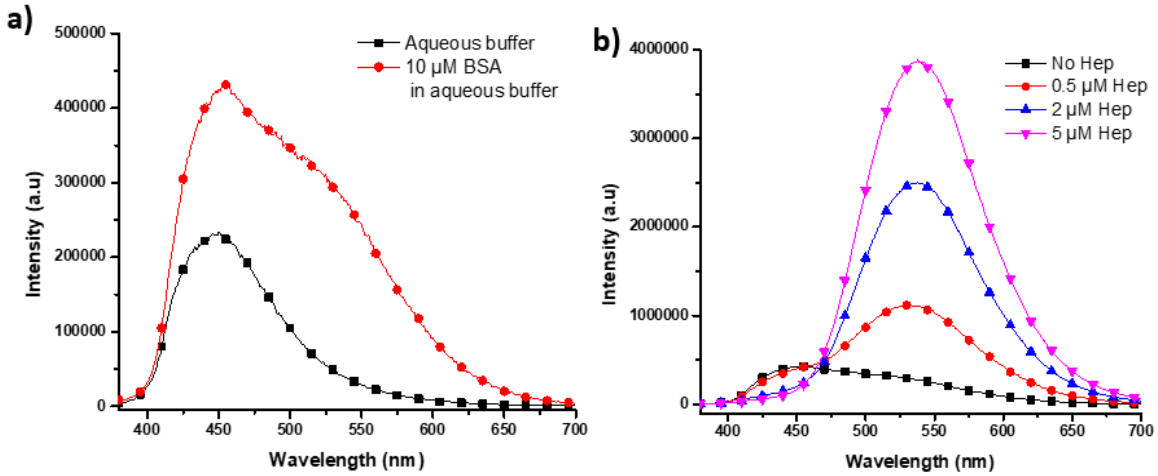


Fig. S23- (a) Emission spectral change of **CS6** (5 μM) upon addition of 10 μM Bovine serum albumin (**BSA**) in aqueous buffer. (b) Emission spectral changes of **CS6** (5 μM) upon addition of heparin in aqueous buffer containing 10 μM of **BSA**.

7. Determination of Activity of Lab Heparin by Activated Partial Thromboplastin Time (aPTT) Test:

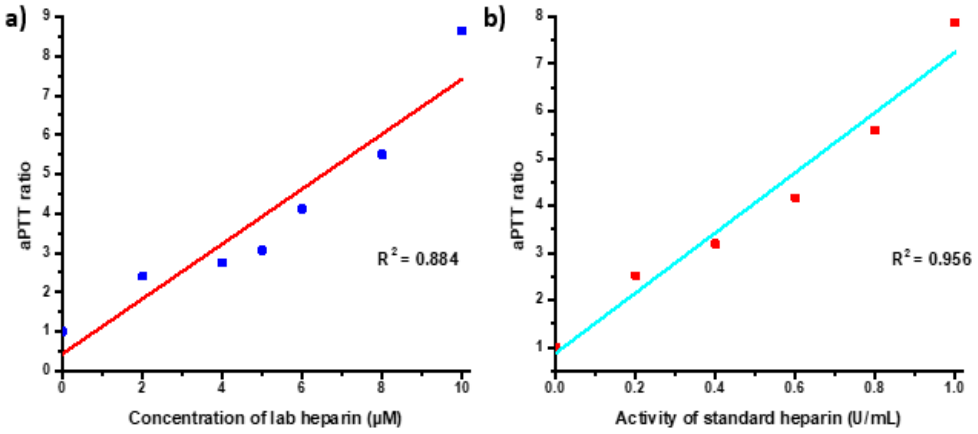


Fig. S24- (a) aPTT ratio (with and without heparin) vs. concentration of lab heparin and (b) aPTT ratio vs. activity of heparin injection in 50% human plasma.

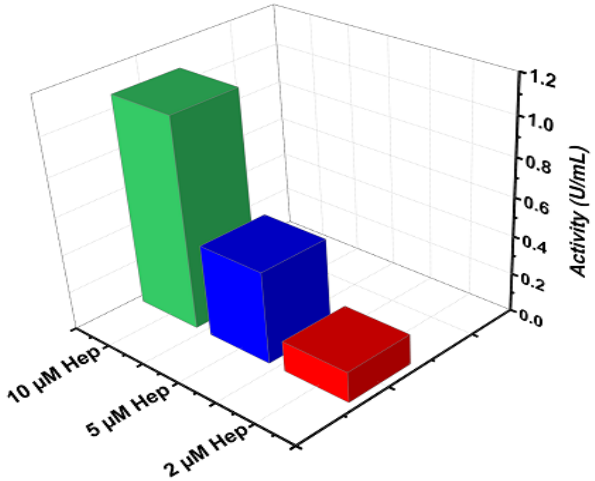


Fig. S25- Activity vs. concentration of lab heparin measured by aPTT test.

Table S1- Activity vs. concentration correlation from emission studies and aPTT

Concentration of Lab Heparin (μM)	**Activity from emission Measurements (U/mL)**	**Activity from aPTT Measurements (U/mL)**
2	-	0.16
5	0.56	0.48
10	1.02	1.08
25	2.38	-

Heparin Activity: Typically, ~0.002 mg/mL of heparin is required to obtain a solution of 1 U/mL activity. Taking the molecular weight of the disaccharide unit as 665.40 g/ mol, this would correspond to a heparin concentration of 3 μM. However, the fluorescence measurements and aPTT tests (Table S1) clearly showed that to obtain a heparin solution of 1 U/mL activity, around 10 μM of the heparin was required. This suggests that only around one-third of all heparin molecules are biologically active.

8. Comparison of Heparin Binding with Protamine Sulfate in buffer:

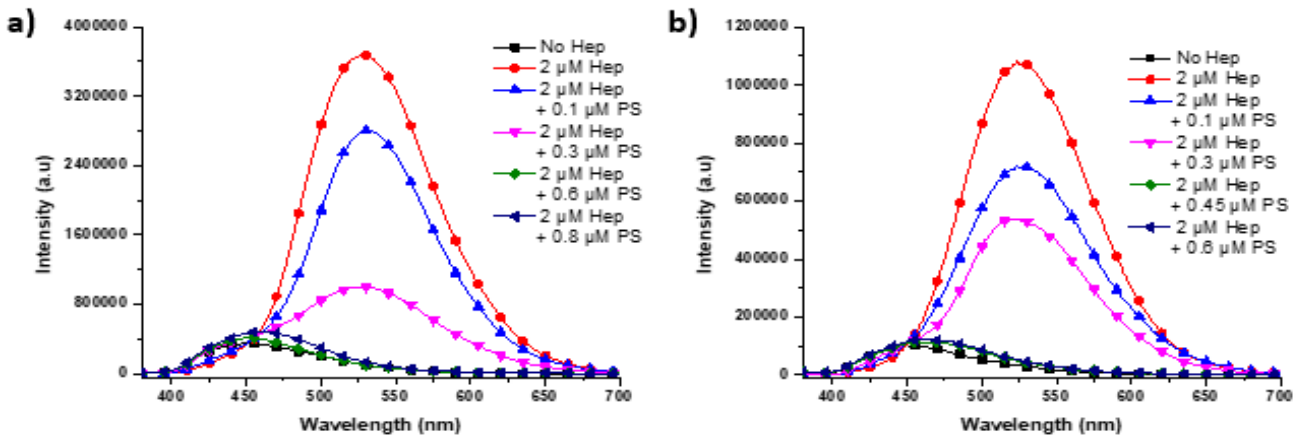


Fig. S26- Emission spectral changes of (a) **CS2** (5 μM) (λ_{ex} = 365 nm) and (b) **CS4** (1.2 μM) (λ_{ex} = 365 nm) upon addition of heparin (2 μM) and subsequent addition of protamine sulfate (**PS**) in aqueous buffer.

9. Time-correlated Single Photon Counting (TCSPC) Measurement:

Table S2- Lifetime (τ_F) of CSNs with and without heparin in aqueous buffer as measured by time-correlated single photon counting (TCSPC).[a]

CS Derivative	Average lifetime (τ_{avg}) in presence of heparin (ns)
CS2 (5 µM)	14.20 (200 nM heparin, 536 nm), 19.21 (5 µM heparin, 536 nm)
CS4 (5 µM)	15.69 (200 nM heparin, 537 nm), 20.57 (5 µM heparin, 537 nm)
CS6 (5 µM)	15.71 (200 nM heparin, 537 nm), 22.38 (5 µM heparin, 537 nm)

[a] Excitation wavelength of the LASER used was 340 nm.

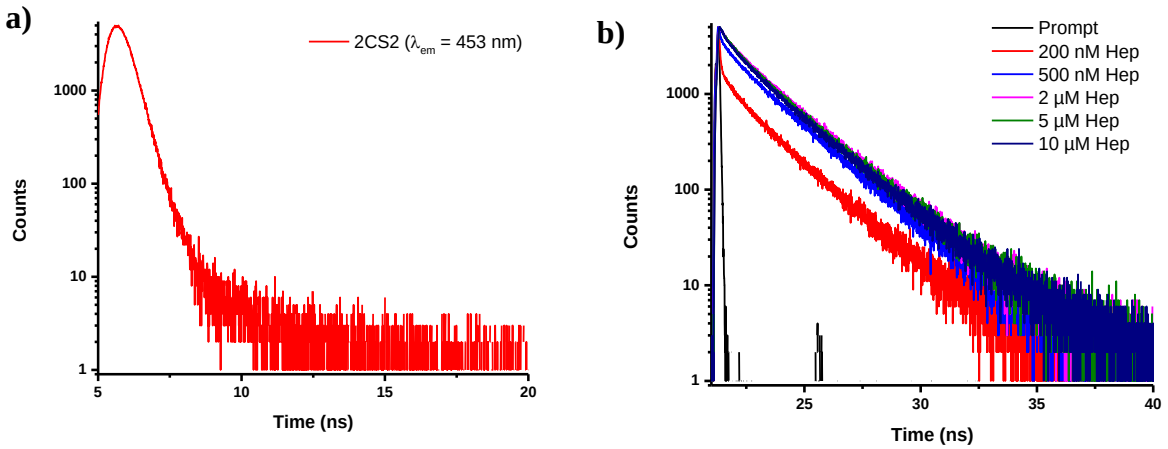


Fig. S27- Time-dependent decay curve of (a) **CS2** (5µM) without heparin (monomeric) (λ_{em} = 453 nm) and (b) with heparin (λ_{em} = 536 nm).

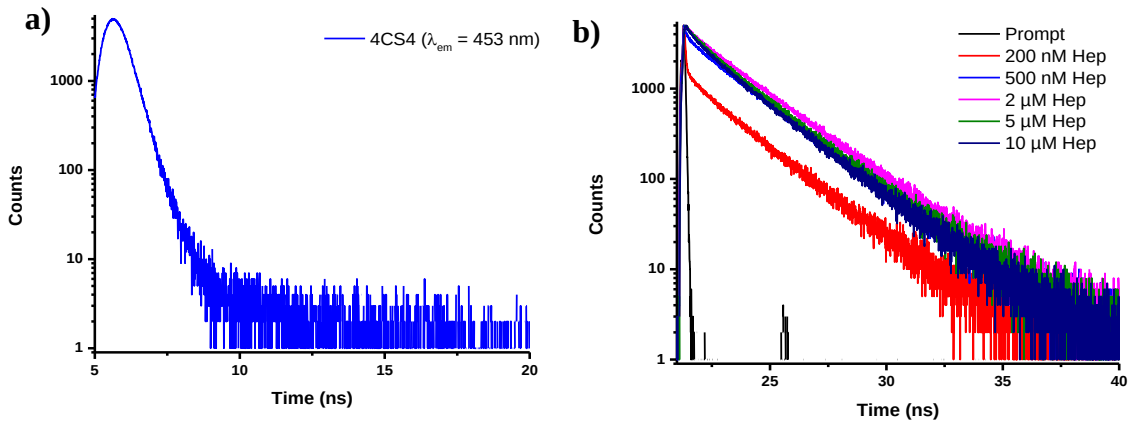


Fig. S28- Time-dependent decay curve of (a) **CS4** (5µM) without heparin (monomeric) (λ_{em} = 453 nm) and (b) with heparin (λ_{em} = 536 nm).

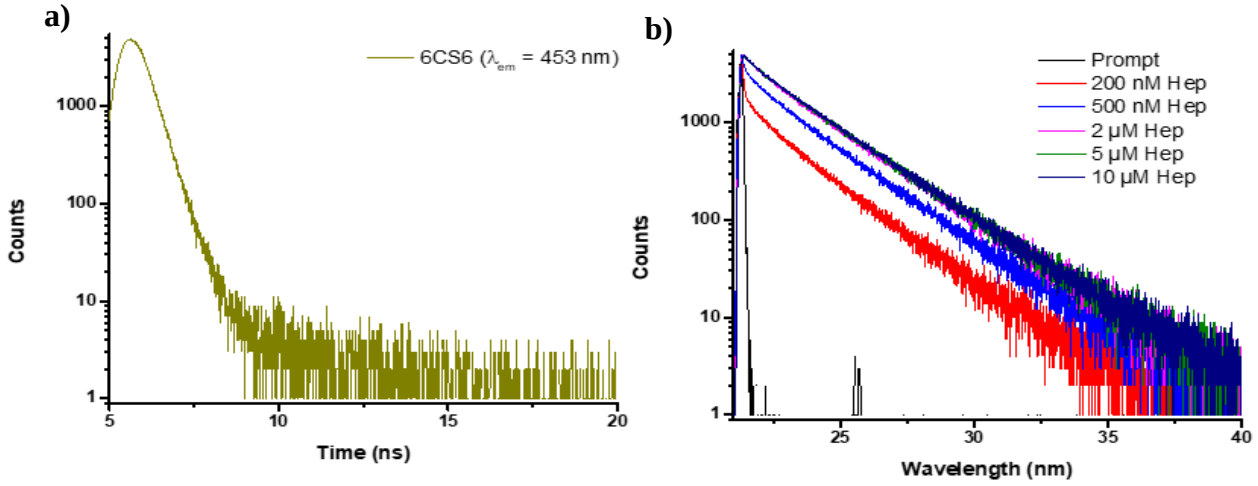
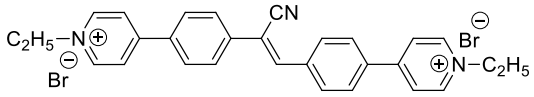
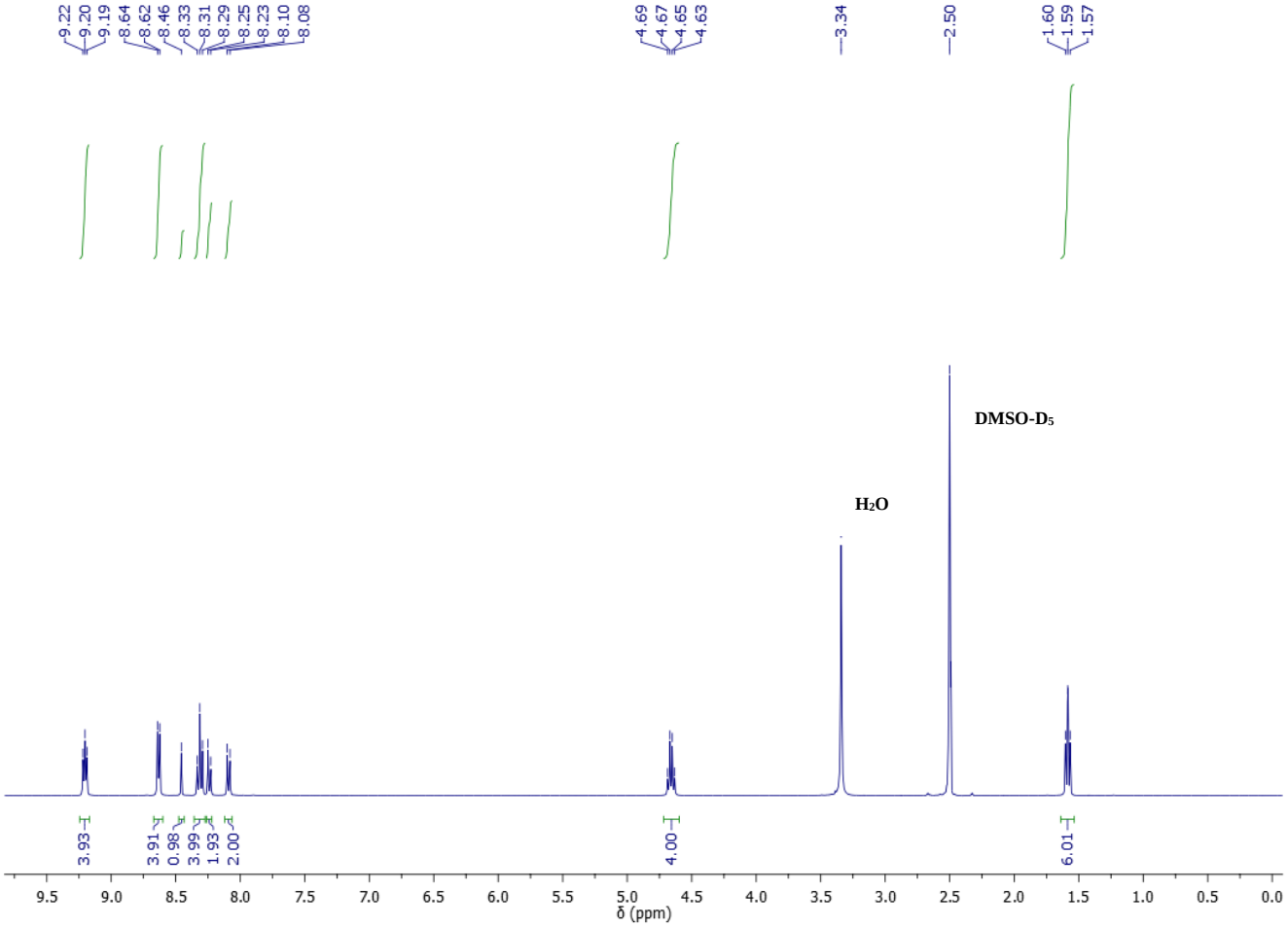


Fig. S29- Time-dependent decay curve of (a) **CS6** (5µM) without heparin (monomeric) (λ_{em} = 453 nm) and (b) with heparin (λ_{em} = 536 nm).

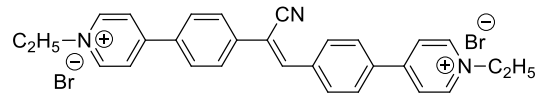
10. NMR Characterization:

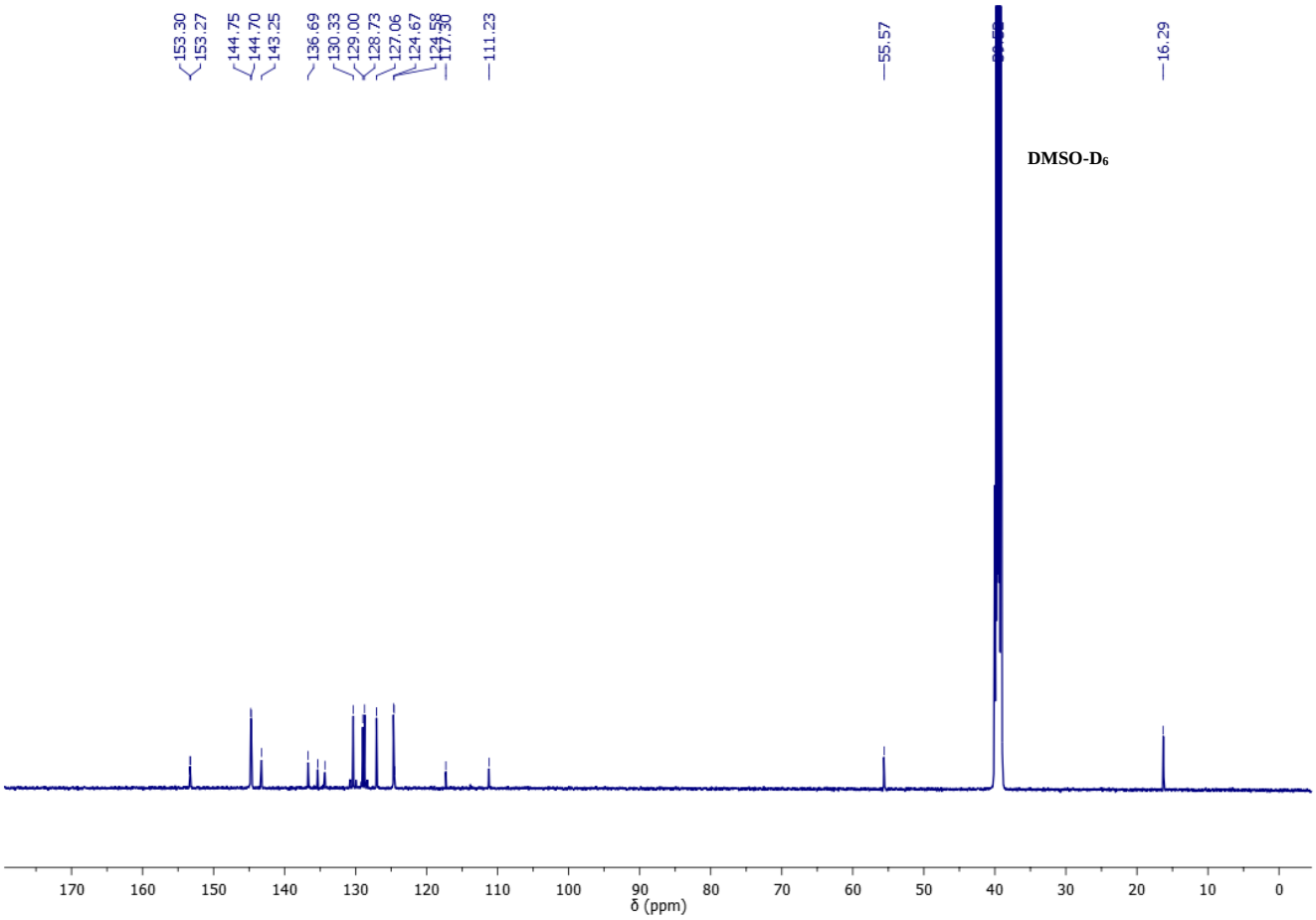
A. ^{1}H NMR spectra of CS2 (400 MHz, DMSO-D_6):



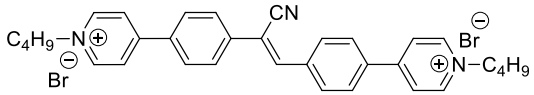


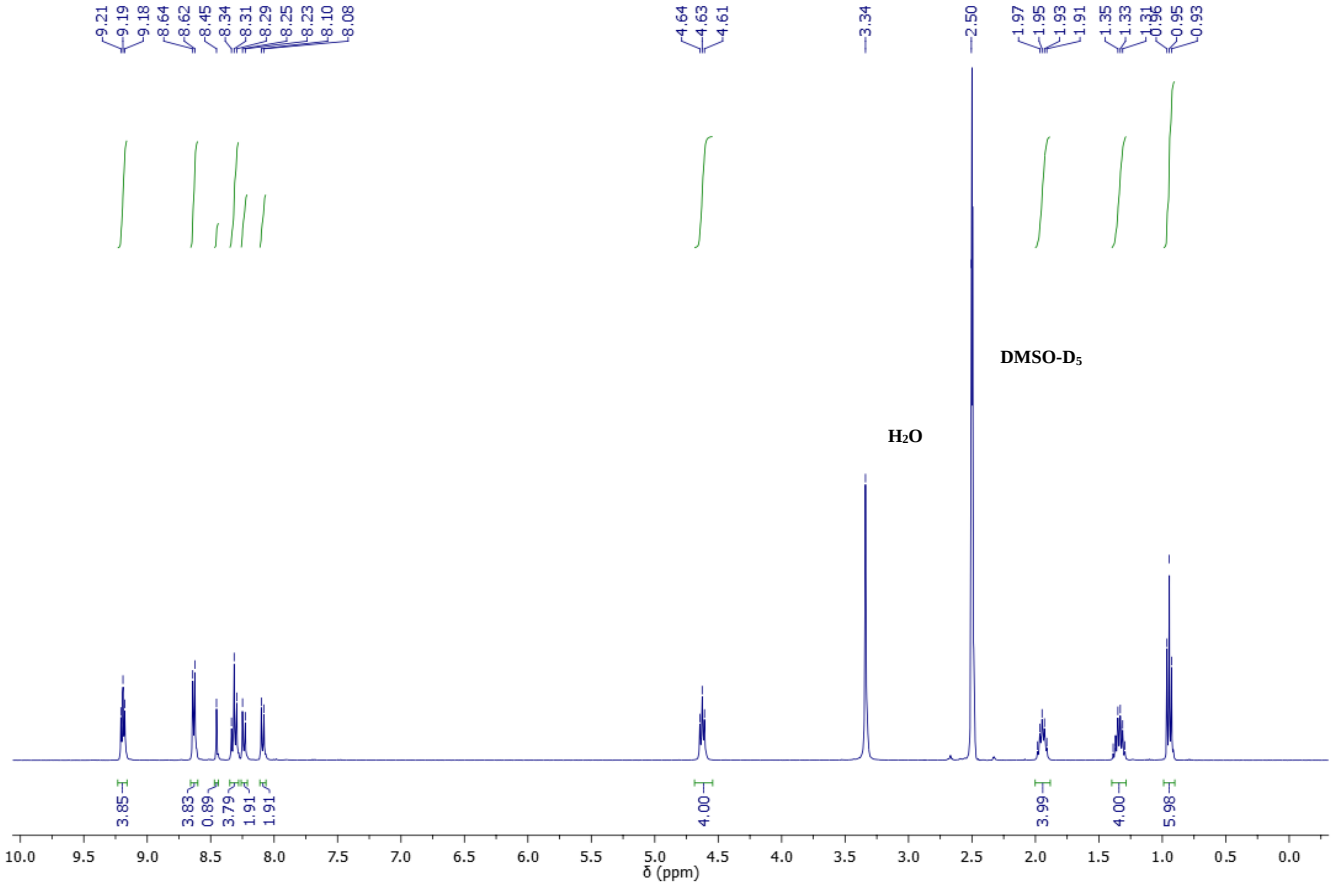
B. ^{13}C NMR spectra of CS2 (125 MHz, DMSO-D$_6$):



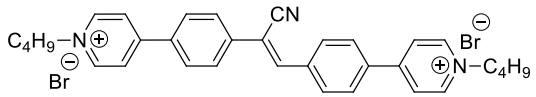


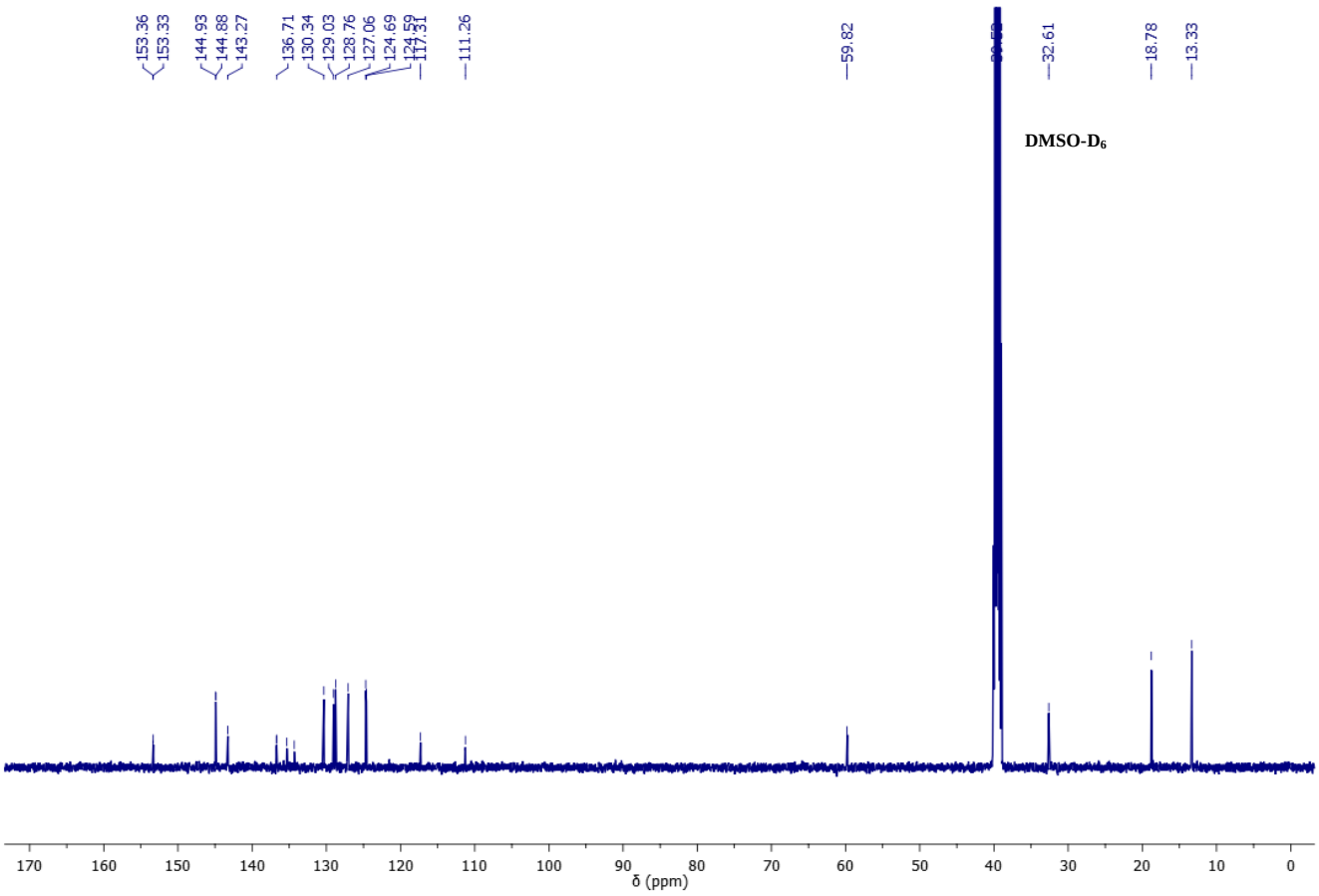
C. ^{1}H NMR spectra of CS4 (400 MHz, DMSO-D$_6$):



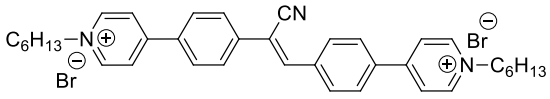


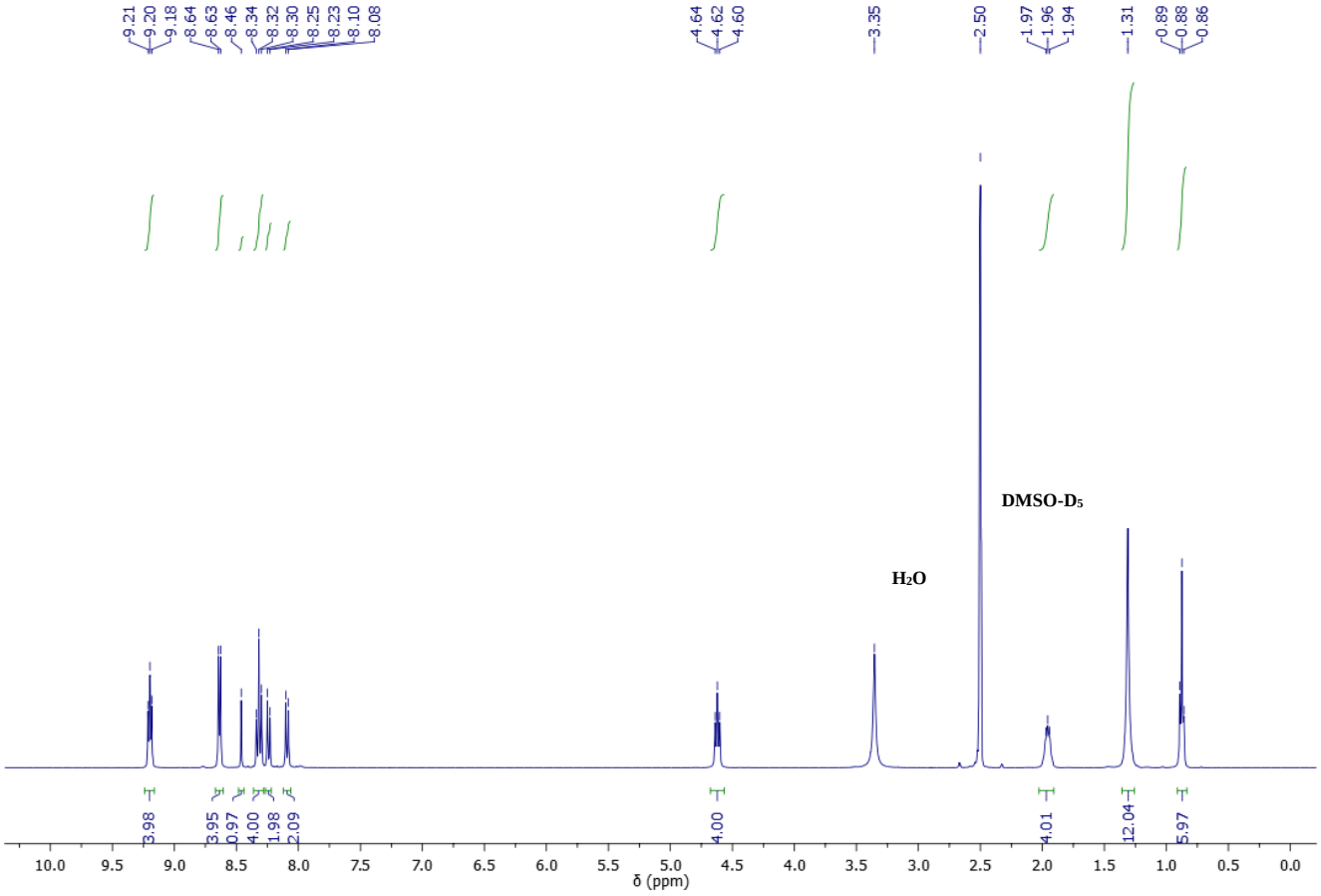
D. ^{13}C NMR spectra of CS4 (125 MHz, DMSO-D$_6$):



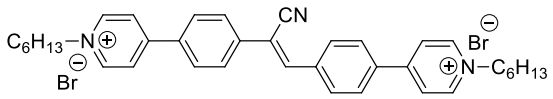


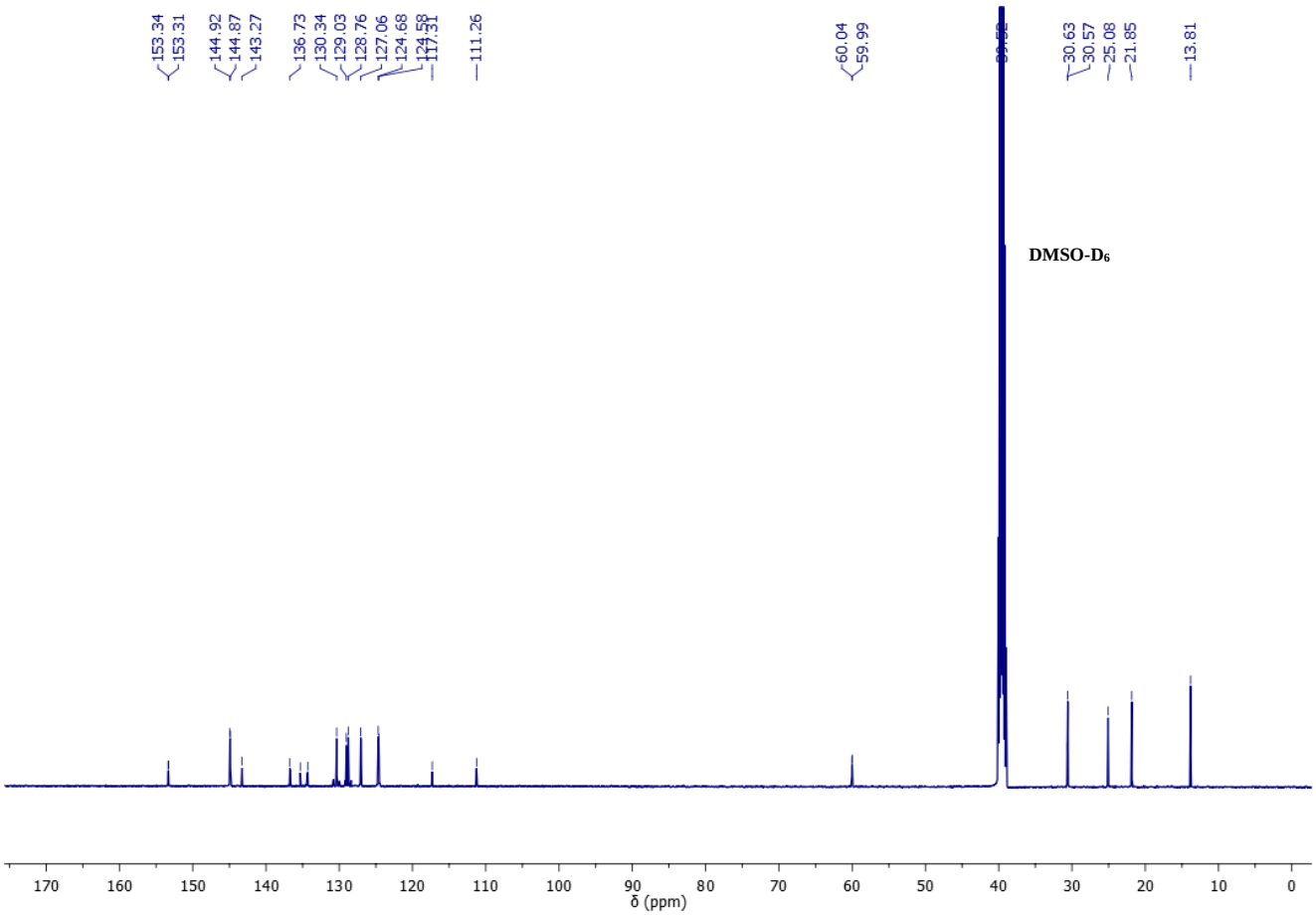
E. ^{1}H NMR spectra of CS6 (400 MHz, DMSO-D$_6$):





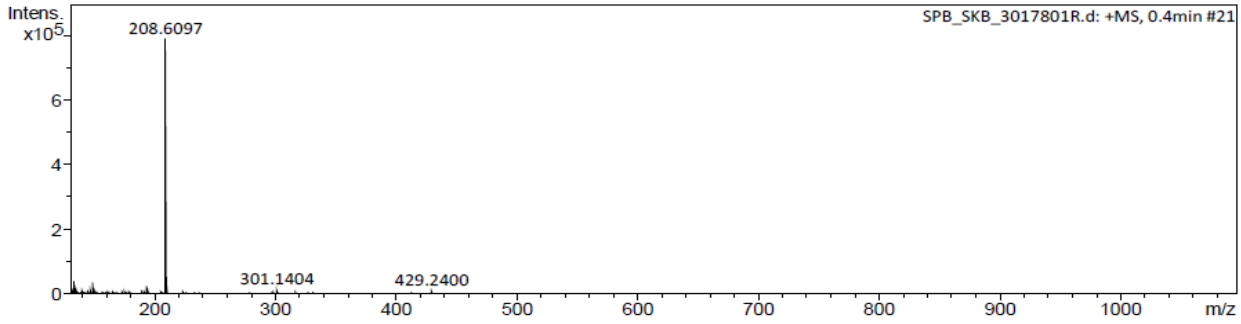
F. ^{13}C NMR spectra of CS6 (125 MHz, DMSO-D$_6$):



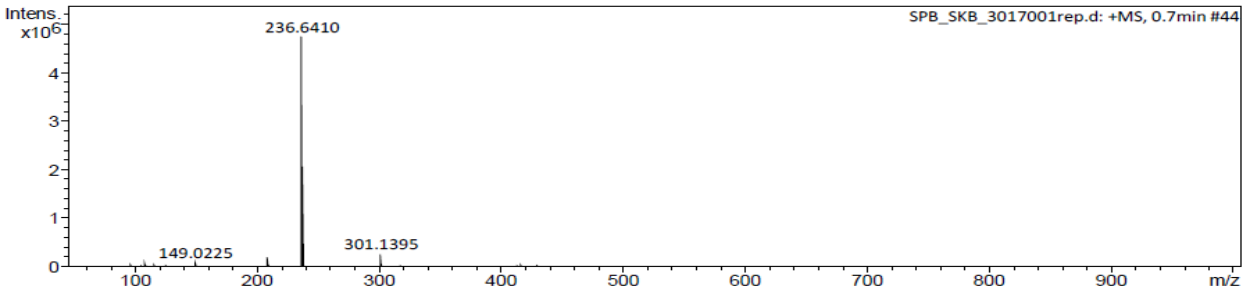


11. Characterization by Mass Spectrometry:

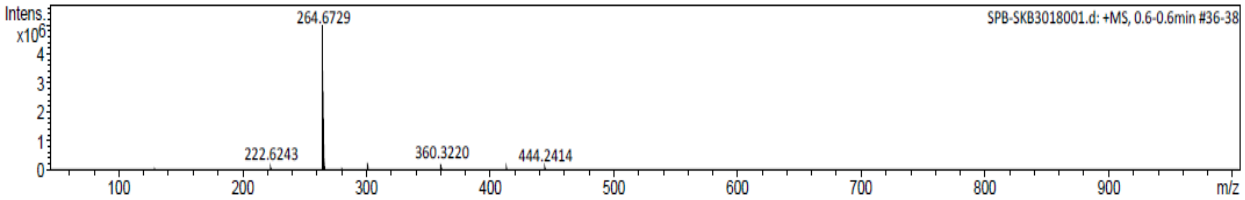
A. CS2:



B. CS4:



C. CS6:



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1 *(a)* Y. You, H. Yang, J. W. Chung, J. H. Kim, Y. Jung and S. Y. Park, *Angew. Chem. Int. Ed.*, 2010, **49**, 3757; *(b)* S. K. Bhaumik and S. Banerjee, *Chem. Commun.*, 2020, **56**, 655.

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