A supramolecular host-guest complex for heparin binding and sensing

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Supporting Information

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1. Materials and Methods

1.1. Synthesis

The resorcin[4]arenes and pillar[5]arene were synthetized according to previously reported procedure.[1–3] Briefly, the functionalizing the upper rim 2-position of the phenyl groups of the resorcin[4]arenes with benzyloxycarbonyl (Boc)-protected amines in the presence of excess formaldehyde and subsequently removing the Boc protections through acid hydrolysis gave the polycationic resorcin[4]arenes (**R4+**, **R8+** and **R16+**).[2] A negatively charged resorcinarene **R4-** was prepared as a control compound.[4] The synthesis of the polycationic pillar[5]arenes (**P10+**) was achieved through quarternization of the ethyl bromine pillar[5]arene with trimethyl amine.[3]

1.2. Nuclear magnetic resonance (NMR) spectroscopy

All NMR samples for host-guest and host-heparin binding studies were prepared in deuterium oxide at constant volume of 500 µl. Additionally, in all sample **P10+** and MO concentrations were kept constant, 2 mM (10 µl 25 mM **P10+**) and 0.2 mM (40 µl 10 mM MO), respectively. In pure heparin sample, concentration was 0.125 mM, and in host-guest-heparin samples concentration ranged between 0.125 and 0.375 mM corresponding to **P10+**:heparin mass ratio 0.5, 1 and 1.5. ^{1}H spectra were measured with Bruker AVANCE 400 MHz instrument at room temperature, and solvent peak was used as reference.

1.3. Dynamic light scattering (DLS)

The hydrodynamic diameter was measured using Zetasizer Nano ZS90 device (Malvern Instruments) with a 4 mW He-Ne ion laser at the wavelength of 633 nm and an Avalanche photodiode detector at an angle of 90°. Experiments were carried out at 25 °C. Zetasizer software (Malvern Instruments) was used to attain the data. Samples for the size measurements were prepared by mixing 8 µl of 10 mg/ml heparin solution to 792 µl of Tris-HCl (10 mM, 150 mM NaCl, pH 7.4). Heparin solutions were titrated with aliquots of 2 µl of 10 mg/ml host solution or with solution of host-guest complex **P10+** (4.1 mg/ml) and MO (0.06 mg/ml). After every addition, the samples were allowed to equilibrate for at least 1 minute. Each titration was carried out three times for individual samples, and all distinct titration points were measured two times. Results presented are the averages with standard deviations of these measurements.

1.4. Methylene blue (MB) displacement assay

Host solutions or host-guest solution of different concentrations was added to a mixture of heparin and methylene blue. To monitor the MB displacement i.e. heparin-host complexation, absorption was measured at wavelengths of 568 and 664 nm with a Biotek Cytation 3 microplate reader. Solution of heparin and MB was prepared by mixing the heparin (0.5 mg/ml) and MB (0.064 mg/ml) solutions in 1:5 volume ratio. Host solutions were prepared by diluting 10 mg/ml stock solution of the host to concentrations of 1.0, 0.8, 0.6, 0.56, 0.5, 0.4, 0.3, 0.2 and 0.1 mg/ml. Host-guest solution of **P10+** and MO was prepared by mixing 60 µl of 0.1 mg/ml MO and 41.6 µl of 10 mg/ml **P10+** solution yielding 1:10 MO:**P10+** molar ratio. This solution was then diluted to correspond **P10+** concentration of 1.0, 0.8, 0.6, 0.56, 0.5, 0.4, 0.3, 0.2 and 0.1 mg/ml. Samples were prepared by mixing 50 µl of host solution with 100 µl of heparin-MB solution. Final MB concentration was 0.034 mg/ml, heparin concentration was 0.07 mg/ml and host concentration ranged between 0.33 and 0.03 mg/ml. All solutions and samples were prepared in 10 mM Tris-HCl buffer at pH 7.4. Absorption at 568 and 664 nm was measured from 96 well plate without lid at room temperature. Ratio of absorbance intensity at 664 nm and 568 nm (A(664)/A(568)) was used to determine the amount of polymer needed for full displacement of MB. Measurements were performed in triplicates and reported as average values with standard deviations.

1.5. Isothermal titration calorimetry (ITC)

For host-guest complexation measurements, host molecule (**R8+**, **P10+** or **R16+**) in 0.05 mM concentration was titrated with 1 mM methyl orange. For host-heparin binding experiments, host molecule **P10+** (5 mM) was titrated into 0.05 mM heparin solution. Additionally, blank titrations into plain buffer were performed and subtracted from the corresponding titration to remove effect from the heats of dilution from the titrant. All solutions were prepared in 10 mM Tris-HCl buffer at pH 7.4. Malvern iTC200 instrument was used to determine the molar enthalpy (ΔH) of binding at 25 °C. Subsequent fitting of the data using Origin software provided the binding constant (K) and the entropy (ΔS). For host-guest fitting one set of sites and for host-heparin measurements sequential fitting model was used. Titrations were run in duplicate.

1.6. Anti-Xa assay

Heparin neutralization with the hosts and protamine in plasma was studied by using a commercial two-stage kit, Biophen Heparin Anti-Xa (221010). Samples were prepared by adding heparin (0.1 mg/ml, 150

mM NaCl) to human plasma followed by hosts in different concentration (150 mM NaCl). Samples were diluted and kit's reagents were reconstructed according to the manufacturer's instructions. Final concentration of heparin was 0.075 units/ml and mass ratio of host-to-heparin ranged between 0.5 and 3. To run the calorimetric assay, 40 µl sample solution and 40 µl antithrombin was added to wells of 96-well microplate and incubated for 2 min. This was followed by addition of factor Xa (40 µl), 2 min incubation, addition of the chromogenic substrate (40 µl), and 2 min incubation. Reaction was stopped by introducing 80 µl of 2 % citric acid, and absorbance at 405 nm was measured immediately using Biotek Cytation 3 microplate reader. Anticoagulant activity is inversely proportional to the absorption intensity and the percentage of neutralization was determined with the help of calibration curve of heparin performed according to the manufacturer's instructions (Figure S3b). Assay was performed three times and averages with standard deviations are presented.

1.7. Methyl orange (MO) host-guest assay

To study host-guest complexation, host solutions were titrated to methyl orange solution. Samples were prepared by adding 60 µl of 0.1 mg/ml MO solution into 1440 µl of Tris-HCl buffer. Titration was done in 5 µl aliquots of 10 mg/ml host solution. All solutions were prepared in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, except **R4+** that was not soluble to buffer with salt.

To investigate heparin's effect on host-guest complexation, heparin was titrated to host-guest complex solution (MO-**P10+**) in Tris-HCl buffer. Samples were prepared by adding 60 µl of 0.1 mg/ml solution of MO and 4.2, 20.8, 41.6 or 62.4 µl of 10 mg/ml solution of **P10+** to the buffer to yield 1:1, 1:5, 1:10 or 1:15 MO:**P10+** molar ratio and 500 µl total volume. This solution was led to stabilize for 5 minutes after which 1 ml of the buffer was added to yield total sample volume of 1.5 ml. To gain comparable heparin:**P10+** mass ratios, all samples were titrated with 5 µl aliquots up to 60 µl as follows: 1:1 samples were titrated with 1 mg/ml heparin solution, 1:5 samples were titrated with 5 mg/ml heparin solution, 1:10 and 1:15 samples were titrated with 10 mg/ml heparin solution. All solutions were prepared in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4.

Calibration curve was prepared by heparinizing 0, 5 and 10 % plasma in different quantities. Samples were prepared by mixing 2-30 µl of 10 mg/ml heparin solution into the plasma-buffer media to yield total volume of 1.5 ml and heparin content of 2.8-42.4 units/ml. All samples were measured as such and after adding 30 µl of MO-**P10+** solution (1:10 molar ratio, **P10+** 4.1 mg/ml, MO 0.06 mg/ml) and stabilizing for 10 minutes. Stabilization time was determined by measuring the samples at time intervals of 0, 5, 10, 15, 20 and 40 min.

Selectivity studies were prepared by titration of MO-**P10+** solution with heparin, hyaluronic acid and chondroitin sulfate. Samples were prepared by adding 60 µl of 0.1 mg/ml MO solution and 41.6 µl of 10 mg/ml **P10+** solution in to Tris-HCl buffer (10 mM, 150 mM, NaCl, pH 7.4) to yield total volume of 1.5 ml. These samples were titrated in 10 µl aliquots up to 100 µl (hyaluronic acid 3.75 mg/ml solution, and chondroitin sulfate as 3.5 mg/ml solution) or 5 µl aliquots up to 30 µl (10 mg/ml heparin solution). All measurements were conducted three times and averages with standard deviations are presented. All samples were measured with Varian Cary50 UV-Vis spectrophotometer by observing absorbance between 300 and 600 nm and by comparing absorption at wavelengths 405 and 470 nm.

2. Dynamic light scattering (DLS)

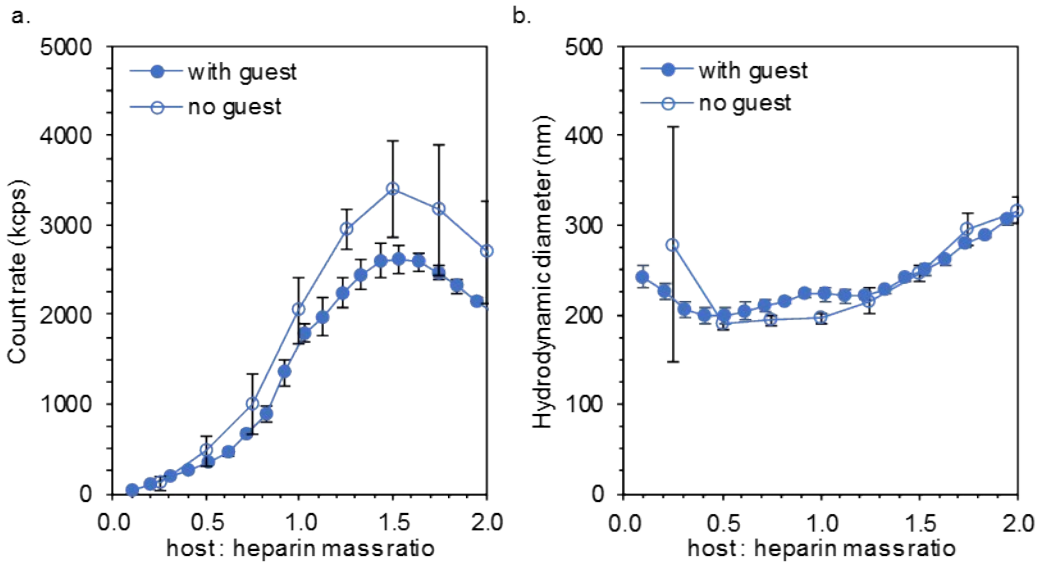

*Figure S1. DLS data showing that the inclusion of the guest molecule, methyl orange, in host **P10+** does not affect the heparin-host complex formation. a) Count rate increases in same way independent of the guest molecule. b) Hydrodynamic diameter is the same for both samples upon titration of host or host-guest mixture.*

3. Methylene blue displacement assay

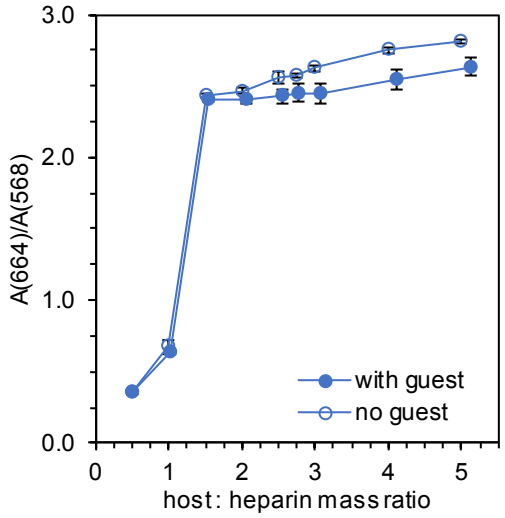

*Figure S2. Based on the methylene blue displacement assay, complex formation between host **P10+** and heparin is not dependent on the presence of the guest molecule, MO.*

4. Isothermal titration calorimetry

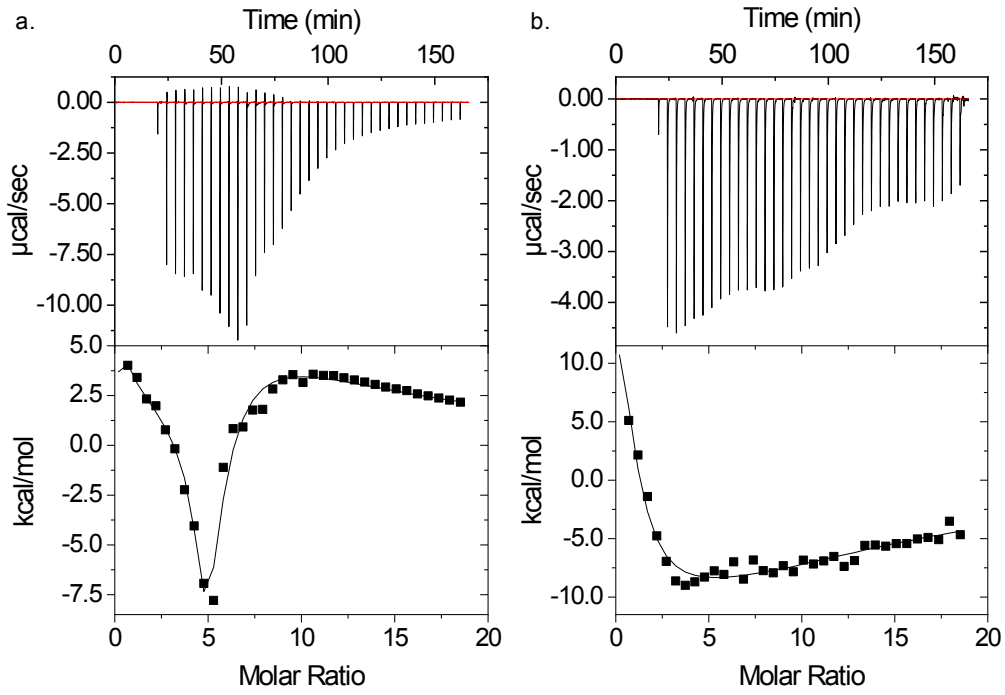

*Figure S3. ITC traces of the titration of host (5 mM) into heparin (0.05 mM) in 10 mM Tris-HCl buffer pH 7.4 at 298 K. Upper graph showing the raw data and the lower graph presenting the integrated data fitted into a sequential binding model after background reduction. (a) Host **R16+** (b) host **R8+**.*

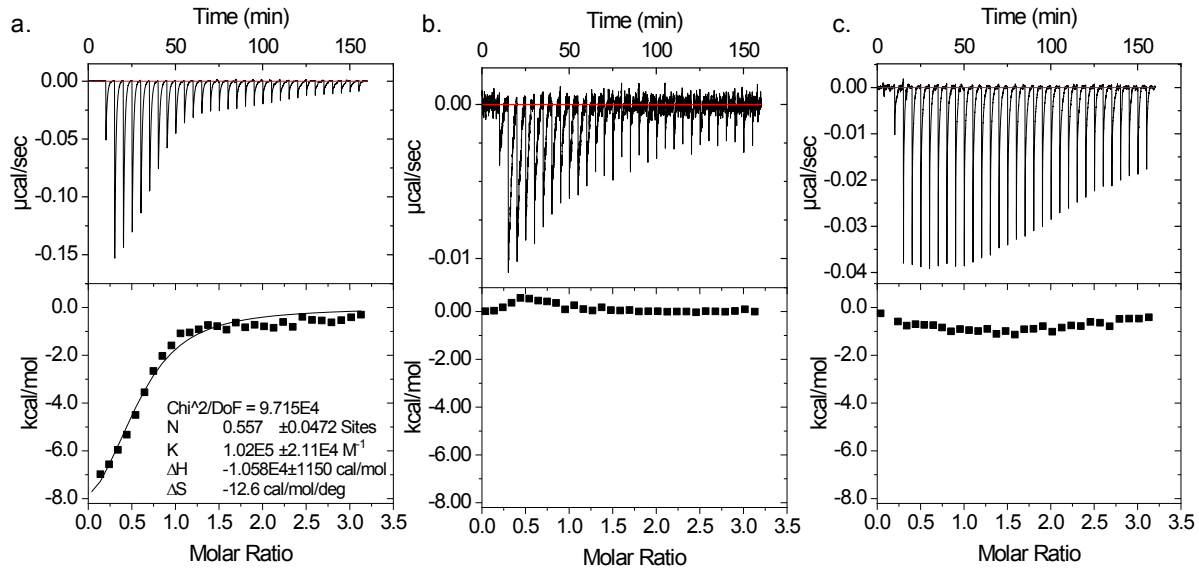

*Figure S4. ITC traces of the titration of MO (1 mM) into guest (0.05 mM) in 10 mM Tris-HCl buffer pH 7.4 at 298 K. Upper graph showing the raw data and the lower graph presenting the integrated data fitted into a one site -model after background reduction. (a) Host **P10+** shows clear host-guest complexation (b) host **R8+** shows no clear guest binding and the data gave no good fit, and (c) host **R16+** shows no clear guest binding and gave no good fit.*

*Table S1. Thermodynamic binding parameters of formed complexes between the host **P10+** (5 mM) and heparin (0.05 mM) by ITC.*

binding site	K x 10^3 (M^{-1})	ΔH (kcal/mol)	TΔS (kcal/mol)	ΔG (kcal/mol)
1	90.4	-5.80E-04	6.8	-6.8
2	81.4	0.8	7.5	-6.7
3	63.9	9.8	16.4	-6.6
4	92.3	-42.7	-35.8	-6.9
5	76	66.3	73	-6.8
6	275	-28.5	-21	-7.4
7	0.1	77.3	80.2	-2.9
sum	679.1	83.0	127.1	-44.1
average	97.0	11.9	18.2	-6.3

*Table S2. Thermodynamic binding parameters of formed complexes between the host **R16+** (5 mM) and heparin (0.05 mM) by ITC.*

binding site	K x 10^3 (M^{-1})	ΔH (kcal/mol)	TΔS (kcal/mol)	ΔG (kcal/mol)
1	2.8	-16.8	-12	-4.7
2	266	34.4	41.7	-7.3
3	631	28.6	36.7	-8.1
4	301	-42.2	-34.6	-7.6
5	20.1	-26.8	-20.9	-5.9
6	1.1	140.9	144.9	-4
sum	1222.0	118.1	155.8	-37.6
average	203.7	19.7	26.0	-6.3

*Table S3. Thermodynamic binding parameters of formed complexes between the host **R8+** (5 mM) and heparin (0.05 mM) by ITC.*

binding site	K x 10^3 (M^{-1})	ΔH (kcal/mol)	TΔS (kcal/mol)	ΔG (kcal/mol)
1	19.5	24.6	30.4	-5.9
2	1.8	-184.7	-180.4	-4.3
3	4.5	171.2	176.2	-5
4	1.3	-289.8	-285.6	-4.2
sum	27.1	-278.7	-259.4	-19.4
average	6.8	-69.7	-64.9	-4.9

The host-heparin binding is a complex system with multiple events, like complexation and precipitation, occurring at the same time. Additionally, both of the components, host and heparin, have multiple binding sites. Additionally, the hosts have different ITC traces most likely due to their different amphiphilic nature. Therefore, the presented data and values are an approximation of the system.

5. Anti-Xa assay

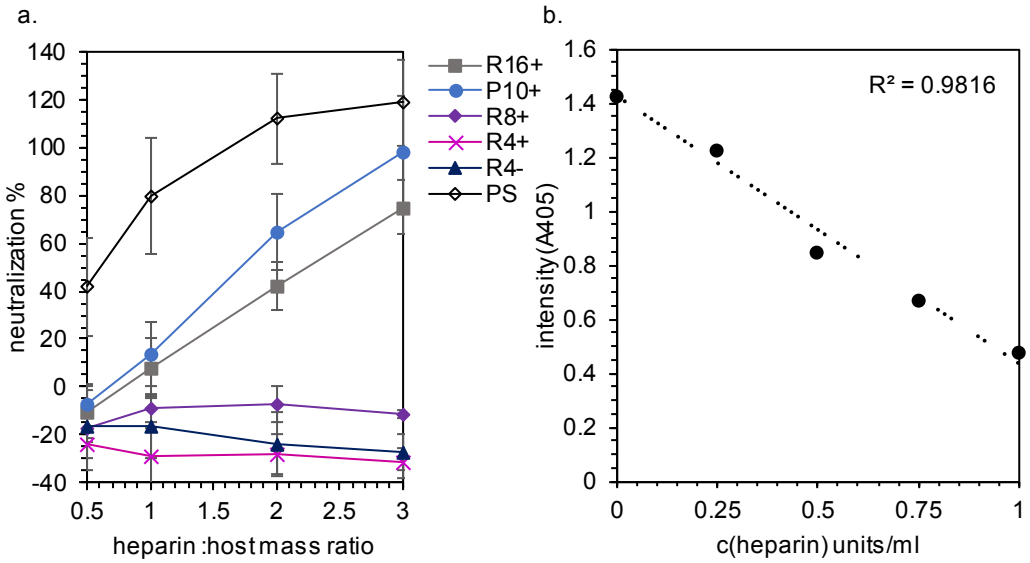

*Figure S5. Anti-Xa assay was used to study heparin neutralization in plasma. a) Assay confirmed that **P10+** and **R16+** effectively bind heparin, but **R8+**, **R4+** and **R4-** show no neutralization activity. b) Calibration curve for the assay.*

6. UV-vis experiments

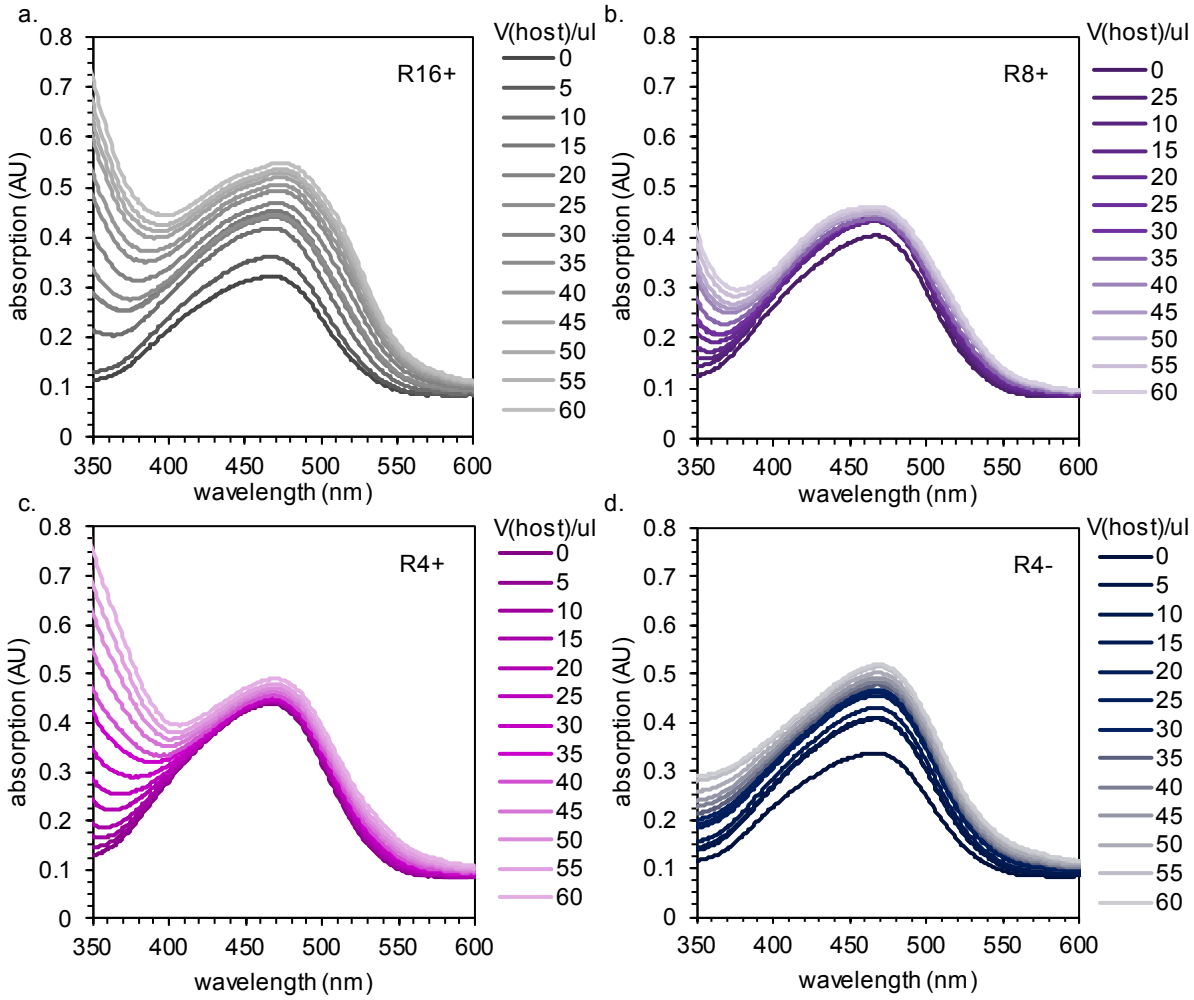

Figure S6. UV-Vis spectra of MO-host titrations show no host-guest complex formation when MO was titrated with a) ***R16+****, b)* ***R8+****, c)* ***R4+*** *and d)* ***R4-****.*

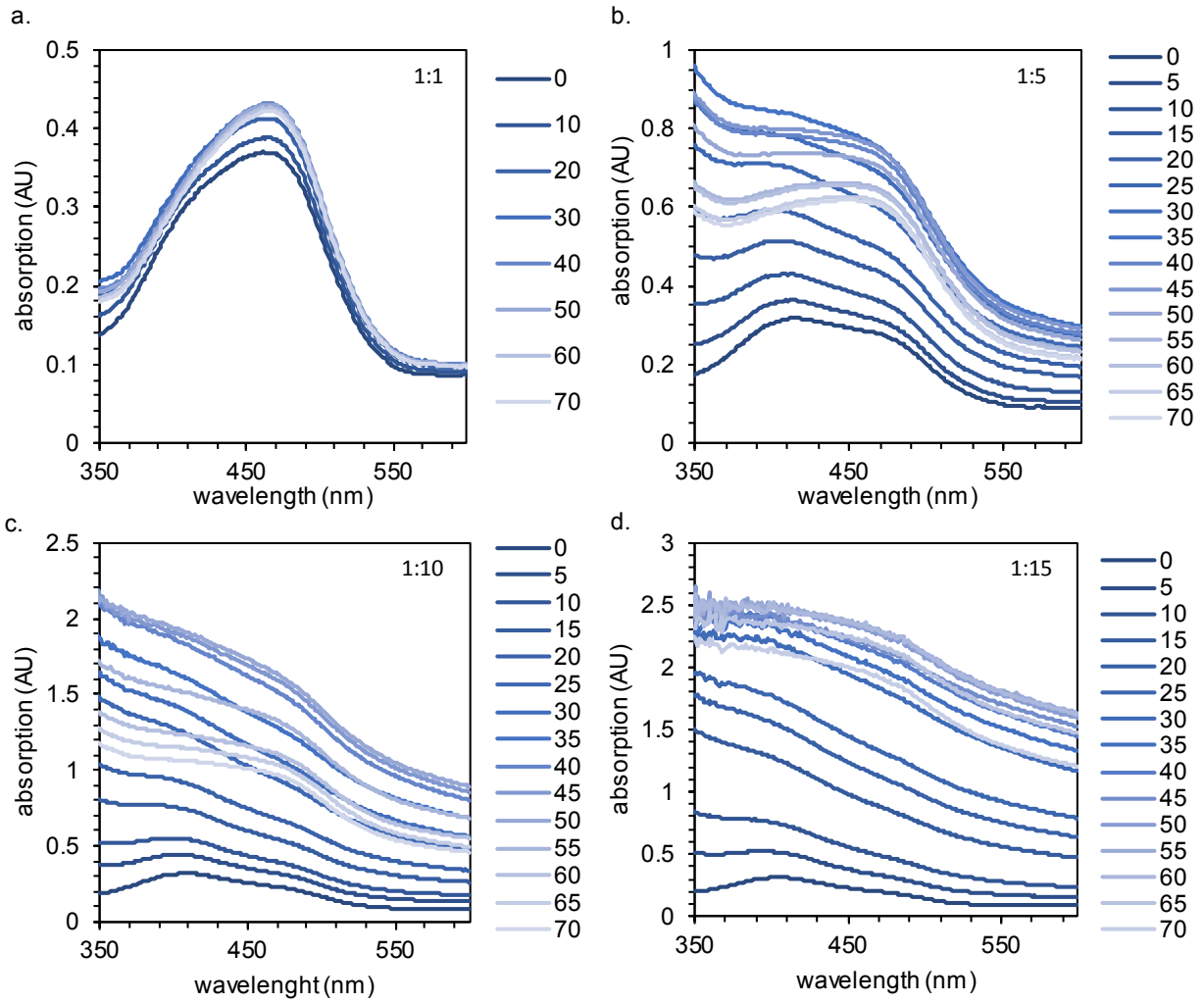

*Figure S7. UV-Vis spectra of different ratios of MO-**P10+** complex titrated with heparin. a) With 1:1 (MO:**P10+**) molar ratio, no clear shift in the absorption maxima is observed. b) With 1:5 molar ratio, more pronounced change from bound state (405 nm) to free state (407 nm) of MO is observed. c) Sample with 1:10 MO:**P10+** ratio shows a clear change. d) 1:15 sample is suffering from poor data quality due to increased cloudiness of the sample upon heparin addition.*

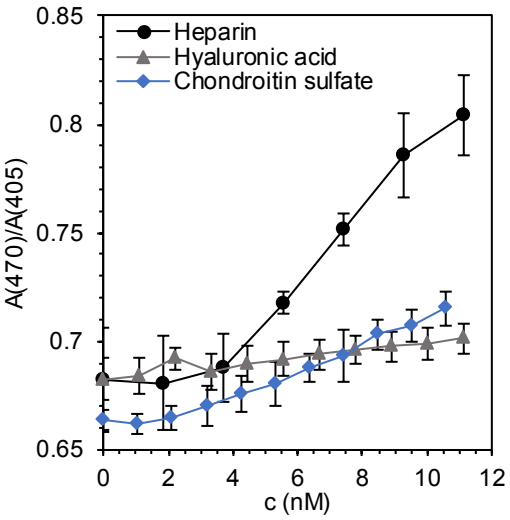

*Figure S8. UV-Vis measurements showing that **P10+** is more selective towards heparin than hyaluronic acid or chondroitin sulfate when measured in 10 mM Tris-HCl buffer (pH 7.4, 150 mM NaCl).*

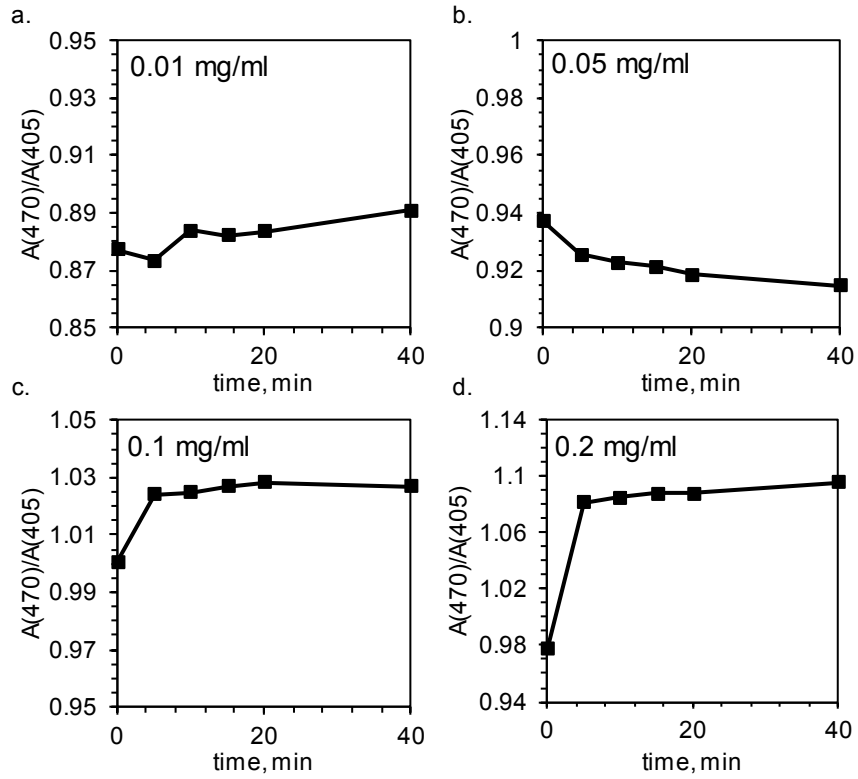

*Figure S9. Stabilization time needed for the calibration curve was evaluated by preparing heparinized samples in 5 % plasma (Tris-HCl 10 mM, pH 7.4) and adding 30 ul MO-**P10+** (1:10 molar ratio) mixture. Samples were measured at time points 0, 5, 10, 15, 20 and 40 minutes.*

7. NMR experiments

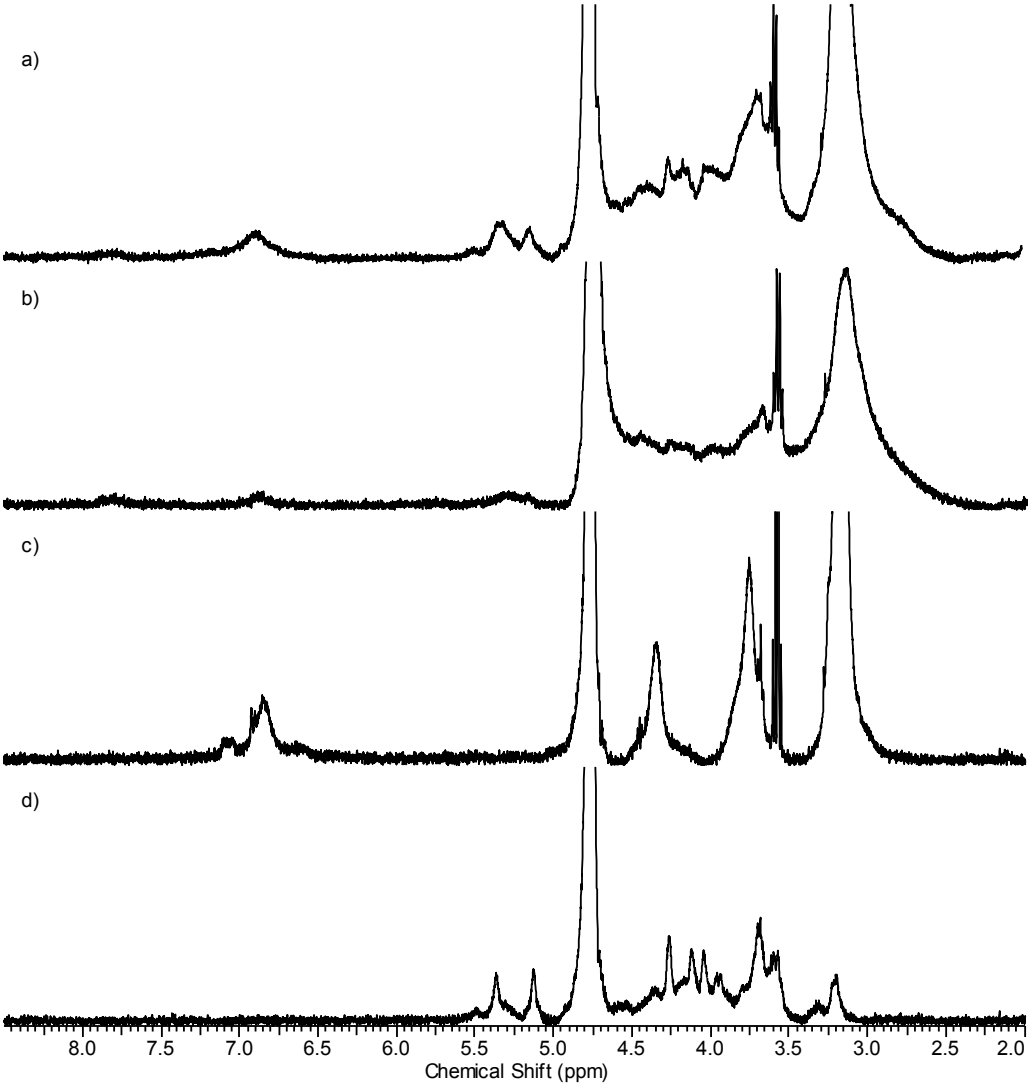

*Figure S10. NMR spectroscopy was used to study the heparin binding with host-guest complex, MO-**P10+**, by keeping the host and guest concentrations constant (2 mM and 0.2 mM, respectively) and adding heparin in the following amounts: a) **P10+**:heparin mass ratio 1:1.5, b) **P10+**:heparin mass ratio 1:1, c) **P10+**:heparin mass ratio 1:0.5, d) heparin only. Suppression of the host signal at 6.86 ppm indicates complex formation along with the phase separation observed in sample preparation. Additionally, appearance of small signal around 7.80 ppm upon heparin addition suggests release of the guest molecule.*

8. References

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