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Differential interactions of conjugated polymer nanoparticles with glycosaminoglycans in synthetic urine

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Experimental

General. Chemicals and solvents were purchased from Fisher Scientific and used as received. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Cambridge, MA). Molecular weights were determined using gel permeation chromatography (GPC) against polystyrene standards on a Shimadzu high performance liquid chromatography (HPLC) system equipped with PLgel 5 μm MIXED-D columns and SPD-20A ultraviolet-visible (UV-vis) detector a flow rate of 1.0 mL/min. UV-Vis spectra were recorded using Varian Cary 50 Bio spectrophotometer. Fluorescence spectra were obtained using a FluoroLog-3 Spectrofluorometer (Jobin Yvon/Horiba). Quantum yields (QYs) were determined using 9,10-diphenylanthracene (QY= 0.9) in cyclohexane as a fluorescence standard. Fourier transform infrared (FT-IR) spectra were recorded on a PerkinElmer Spectrum 100 FT-IR Spectrometer. Fine powders from lyophilized samples were directly mounted on an attenuated total reflection (ATR) cell of the spectrometer. The purification of the CPNs was conducted using an Ultrafiltration Stirred Cell (Millipore) with a 10 kDa molecular weight cut-off (MWCO) membrane (Ultracel ultrafiltration disc). Nuclear magnetic resonance (NMR) spectra for the conjugated polymers (CPs) were recorded on a 400 MHz Avance Bruker NMR spectrometer. Chemical shifts were reported in parts per million (ppm) for ^{1}H NMR on the δ scale based on the middle peak (δ = 2.50 ppm) of the dimethylsulfoxide (DMSO)-d_6 solvent as an internal standard. NMR spectra for the conjugated polymer nanoparticles (CPNs) were recorded on a 600 MHz

Avance Bruker NMR spectrometer using a 5 mm BBI probe at 298 K. The 600 MHz NMR spectrometer is equipped with a gradient system capable of producing magnetic field pulse gradients in the z-direction of about 50 G cm^{-1} and allowing for water peak suppression [δ = 4.79 ppm in deuterium oxide (D_2O)]. Chemical shifts were reported in parts per million (ppm) for ^1H NMR on the δ scale based on the middle peak (δ = 4.79 ppm) of D_2O solvent as an internal standard. Graphs were plotted using Origin 9.1 software (OriginLab, Northampton, MA, USA).

Polymer synthesis.

General procedure. A Schlenk flask was charged with aryl halide monomer (1.0 equiv) and diacetylene monomer (1.0 equiv for **P2**, 0.9 equiv for **P3**), and cystine linker when applicable (0.1 equiv for **P3**) along with $Pd[(PPh_3)_2Cl_2]$ (0.1 equiv) and CuI (0.05 equiv). The Schlenk flask was evacuated and filled with N_2 three times. A solution of anhydrous dimethylformamide (DMF) (3 mL) and morpholine (1 mL) was degassed, and 2 mL of the mixed solution was transferred to the Schlenk flask using a cannular needle. The reaction mixture was heated at 50 °C for 18 h. The solution was then cooled to room temperature and transferred dropwise to cold ethyl ether, resulting in precipitation. After centrifugation (5 min, 4000 rpm), the supernatant was decanted, and the precipitate was redissolved in DMF (1 mL). The resulting polymer was characterized using GPC by diluting an aliquot of polymer solution in 1 mL of HPLC grade tetrahydrofuran (THF) and filtered through 0.45 μm polytetrafluoroethylene (PTFE) syringe filter prior to injection. The absorption and emission profiles were measured in a 10 mm quartz cuvette (2 mL) using a diluted aliquot of the polymer solution in DMF. The material was then reprecipitated in pure ether, the supernatant was decanted, and the precipitate was purified two

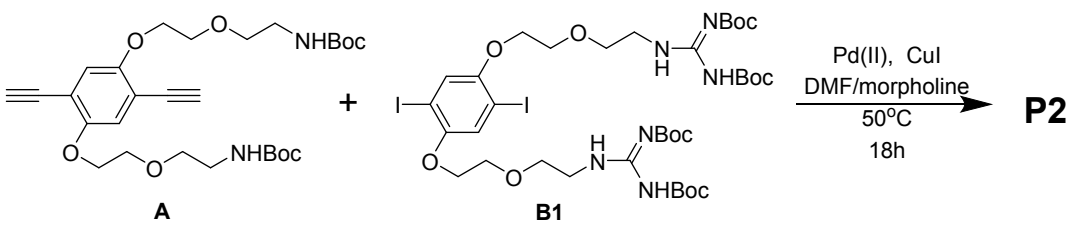
more times. The precipitated polymer was allowed to dry under high vacuum for 4 hours prior to FT-IR and ^{1}H NMR characterization.

CPN fabrication. Boc-deprotection of the polymer was carried out by adding the polymer solution in DMSO-d_6 to a stirred mixture of trifluoroacetic acid (2 mL) and acetic acid (2 mL) and allowed to stir at room temperature for 2 days. The mixture was then diluted by addition of acetic acid (10 mL), and added dropwise (2 drops/s) to 500 mL water (18 Ω) while stirring. Using a solvent-resistant stir cell fitted with a 10 kDa-MWCO membrane, the solution was concentrated to approximately 10 mL, and dialyzed against 2 L of water. The solution was subsequently filtered through a cellulose syringe filter (0.45 μm), characterized, and stored for future use.

P1: Detailed monomer and polymer synthesis, CPN fabrication and characterization is described elsewhere.[1]

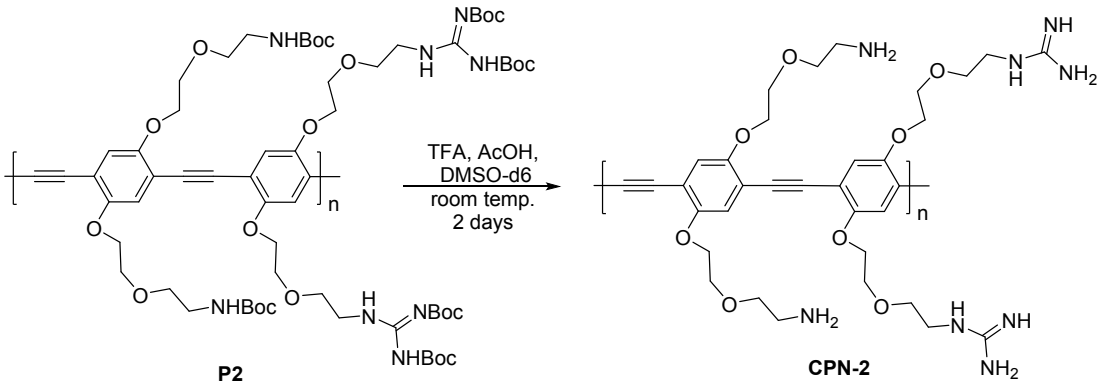
P2: Detailed monomer synthesis and characterization of monomers **A** and **B1** is described elsewhere.[1,2] Using the general procedure described above, the polymerization of monomer **A** (7.8 mg, 0.0147 mmol) and monomer **B1** (15.0 mg, 0.0147 mmol) in the presence of $Pd[(PPh_3)_2Cl_2]$ (1.4 mg, 0.00147 mmol) and CuI (0.1 mg, 0.000735 mmol) yielded **P2** (11.5 mg, 0.00866 mmol, 58.9%), see scheme 1. CPN fabrication was carried out as described in the general procedure to yield **CPN-2**, see scheme 2.

Scheme 1. Synthetic route to **P2**.



P2: ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.19 (s, 0.15H), 12.38 (s, 1.18H), 9.00 (s, 0.15H), 8.19 (s, 1.16 H), 7.11 (d, 2.07H), 6.70 (s, 1.23), 4.17 (br m, 4.39H), 3.85-3.76 (br m, 7.09H), 3.56-3.45 (br m, 13.5 H), 3.08 (s, 2.88H), 1.44-1.34 (d, 27.0 H). FT-IR (neat): 3351, 2973, 2965, 2928, 2256, 1709, 1630, 1583, 1505, 1455, 1411, 1366, 1301, 1273, 1245, 1146, 1113, 1050, 1023 cm^{-1}. GPC: M_w = 23,618 Da, M_n = 14,079 Da, PDI = 1.68. UV-Vis (DMF) λ_{max} = 439 nm, fluo λ_{max} (430 nm ex) = 475 nm, QY = 20%.

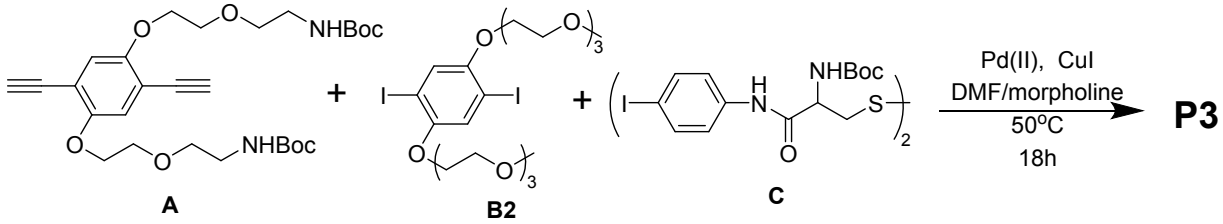
Scheme 2. Boc-deprotection of **P2** to yield **CPN-2**.



CPN-2: ^{1}H NMR (600 MHz, D_2O, δ): 7.06 (br, 2H, Ar-H), 4.30-3.19 (br, 11.87H, $CH_2CH_2OCH_2CH_2$), 1.16 (s, 0.02H); FT-IR (Neat): ν = 3310, 2863, 1560, 1419, 1359, 1301, 1273, 1199, 1110, 1053, 1020 cm^{-1}; UV-vis (H_2O): λ_{max} = 411 nm; fluo λ_{max} (400 nm ex) = 479 nm; QY = 0.64%.

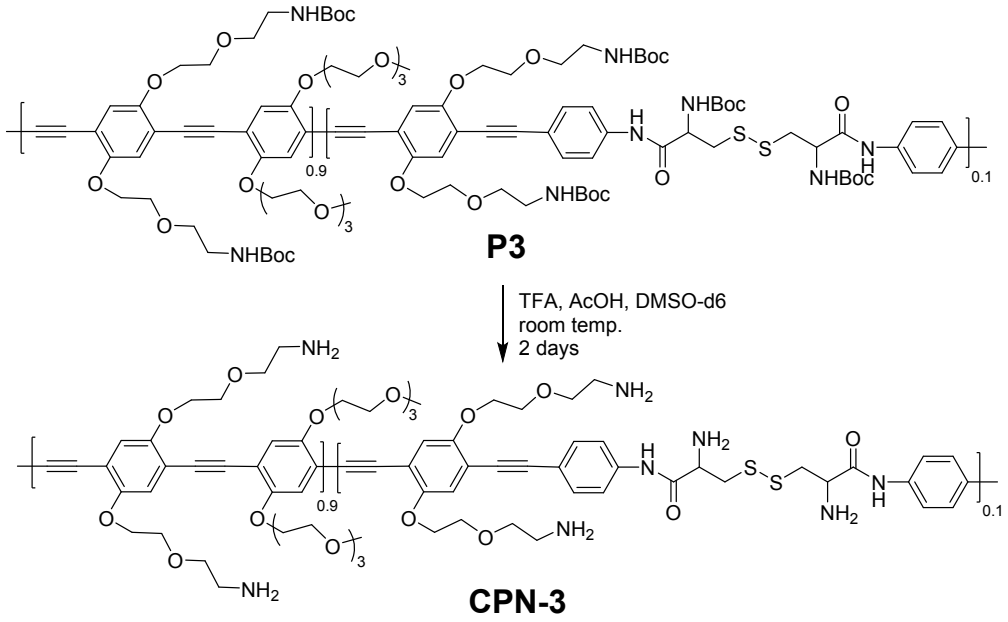
P3: Detailed monomer synthesis and characterization of monomers **B2** and **C** is described elsewhere.[1,2] Using the general procedure described above, the polymerization of monomer **A** (20.0 mg, 0.0375 mmol), monomer **B2** (22.1 mg, 0.0337 mmol), and monomer **C** (3.2 mg, 0.00375 mmol) in the presence of Pd[(PPh_3)Cl_2] (2.6 mg, 0.00375 mmol) and CuI (0.4 mg, 0.00187 mmol) yielded **P3** (17.9 mg, 0.0225 mmol, 60.0%), see scheme 3. CPN fabrication was carried out as described in the general procedure to yield **CPN-3**, see scheme 4.

Scheme 3. Synthetic route to **P3**.



P3: ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.70 (s, 0.11H), 7.71 (s, 0.10H), 7.51 (s, 0.13H), 7.16 (s, 2.10), 6.70 (s, 1.06H), 6.39 (s, 0.12), 5.75 (s, 0.38), 4.00 (br m, 4.00H), 3.80 (br m, 4.87), 3.66 (br m, 2.27H), 3.19 (br m, 3.44H), 1.35 (s, 11.18 H). FT-IR (neat): 3002, 2778, 2505, 1654, 1486, 1462, 1429, 1362, 1321, 1244, 1176, 1133, 1052, 1043 cm^{-1}. GPC: M_w = 23,181 Da, M_n = 14,489 Da, PDI = 1.59. UV-Vis (DMF) λ_{max} = 439 nm, fluo λ_{max} (400 nm ex) = 511 nm, QY = 39%.

Scheme 4. Boc-deprotection of **P3** to yield **CPN-3**.



CPN-3: ^{1}H NMR (600 MHz, D_2O, δ): 7.00 (br, 2H, Ar-H), 4.24-3.26 (br, 17.34H, $CH_2CH_2OCH_2CH_2$); FT-IR (Neat): ν = 3357, 2873, 1581, 1444, 1420, 1351, 1302, 1199, 1093, 1047 cm^{-1}; UV-vis (H_2O): λ_{max} = 422 nm; fluo λ_{max} (400 nm ex) = 464 nm; QY = 0.36%.

P4: Detailed monomer and polymer synthesis, CPN fabrication and characterization is described elsewhere.[1,3]

CPN/GAG complexation. Sodium hyaluronate (HA) was purchased from Lifecore (MW 100 K) and used as received. A stock solution was prepared by dissolving 2.0 mg of HA in 1 mL of deionized water. Heparin sodium was purchased from Acros Organics and used as received. A stock solution was prepared by dissolving 4.0 mg of HS in 1 mL of deionized water. Chondroitin sulfate A (CS) and chondroitin sulfate B (dermatan sulfate, DS) were purchased from Sigma Aldrich and used as received. Stock solutions of CS and DS were prepared by dissolving 2.0 mg and 1.0 mg, respectively, in 1 mL of deionized water. CPN and GAG were mixed and allowed to incubate for 30 minutes prior to measurements. For consistency between the CPNs, the concentration was adjusted to give an optical density of 0.1, which corresponds to approximately 10 μM (based on polymer repeating unit). The GAG concentration used was 30 μM (based on GAG repeating unit). Samples were prepared in deionized water, unless otherwise stated.

Determination of hydrodynamic diameters of CPNs. Light scattering measurements were performed with a LM10 HS (NanoSight, Amesbury, United Kingdom), equipped with a sCMOS camera, sample chamber with a 488 nm blue laser, and Viton fluoroelastomer o-ring. The samples were prepared in similar manner for absorption and emission measurements using water (18 Ω) filtered through 0.45 μm PTFE syringe filters. The samples were injected into the sample chamber with 1 mL sterile syringes (Restek Corporation, Pennsylvania, USA) until the liquid

reached the tip of the nozzle. All measurements were performed at 25°C using a LM14C temperature controller (NanoSight, Amesbury, United Kingdom). Each sample was measured three times.

Determination of zeta potentials of CPNs. Dynamic light scattering measurements were performed by Zetasizer nano–ZS (Zen 3600, Malvern Instruments Ltd.) using a folded capillary cell (Catalog # DTS1060), at room temperature. The samples were prepared at approximately 0.5 mM in water (18 Ω), which was filtered through 0.45 μM PTFE syringe filter. Each sample was measured six times.

Hierarchical cluster analysis (HCA) of emission spectra. The commercially available statistical software JMP® (version 11) was used for analysis. For simplicity, one CPN emission data set in water (triplicate samples) was used for HCA to demonstrate role of functional group in differentiation. The default distance calculation method, Ward's, was used for cluster distances.

CPN in synthetic urine. Surine™ was purchased from Dyna-Tek Industries, Inc. (product #720) and used as received. Surine™ is a urine simulant, with a proprietary formula, which contains creatinine and urea, and was used as media for CPN/GAG measurements. For the differentiation application and to simulate detection of GAGs present in urine, GAGs (100 nM) were mixed in Surine™, then CPN was added to the GAG-containing urine simulant and allowed to incubate for 30 minutes prior to measurements.

LDA of emission spectra. The commercially available statistical software JMP® (version 11) was used for analysis. The data included in the training matrix were the emission spectra ratios of CPN to CPN+GAG in urine simulant. The matrix training set was 4 CPNs x 4 GAGs x 3

replicates, an input of 48 data sets (spectrum ratios). The discrimination method used was the linear, common covariance method.

Supporting data

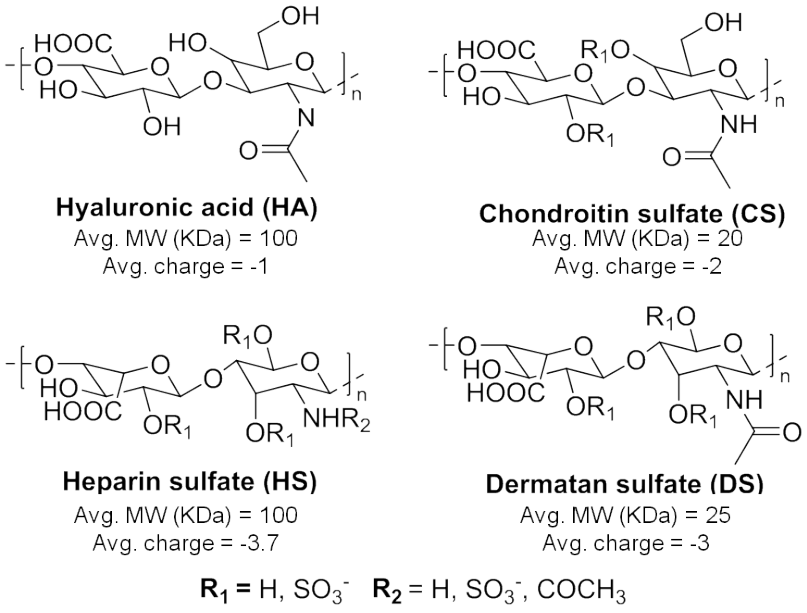


Fig. S-1 Chemical structures of glycosaminoglycans.

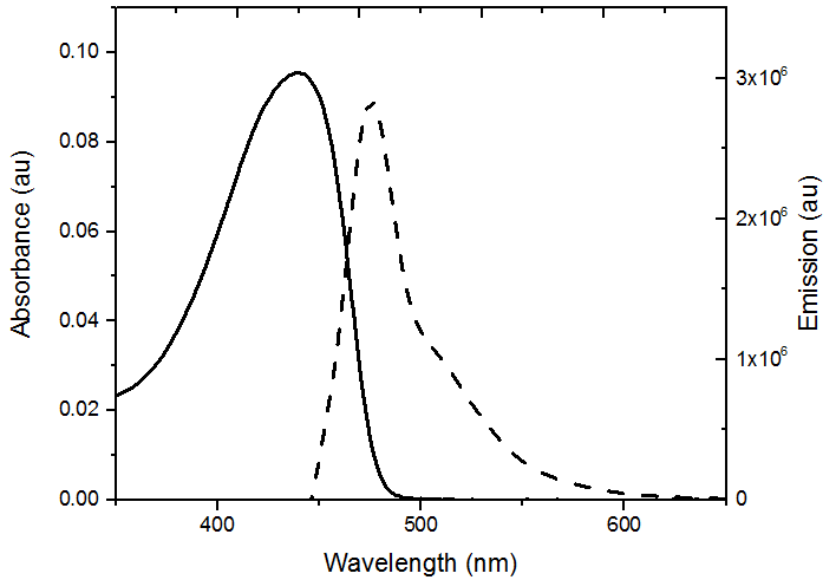


Fig. S-2 Absorption and emission spectra for **P2** in DMF (Excitation = 430 nm, slit widths = 3 nm, integration time = 0.1 s).

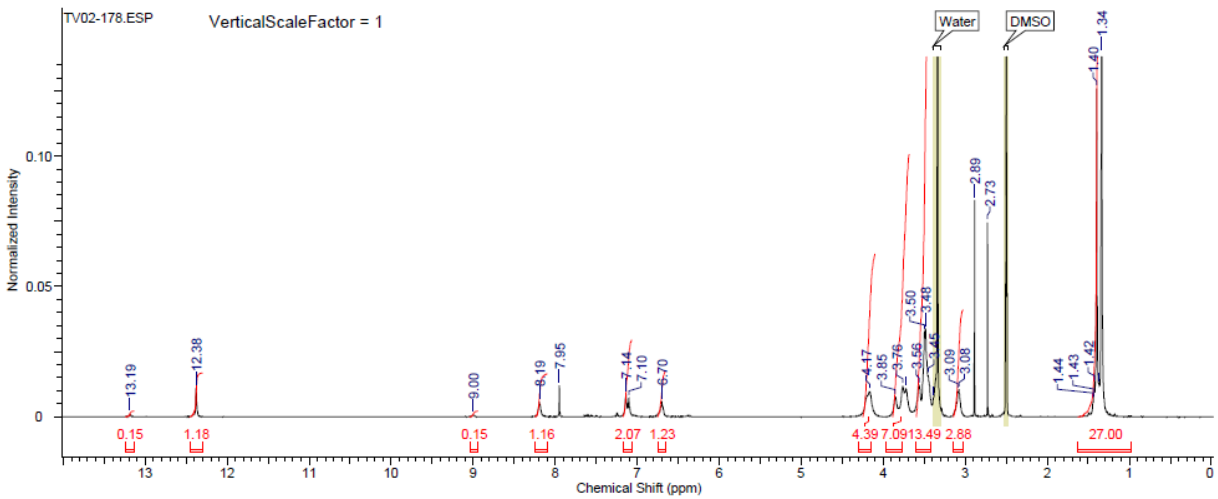


Fig. S-3 ^{1}H NMR (400 MHz) of **P2** in DMSO-d6.

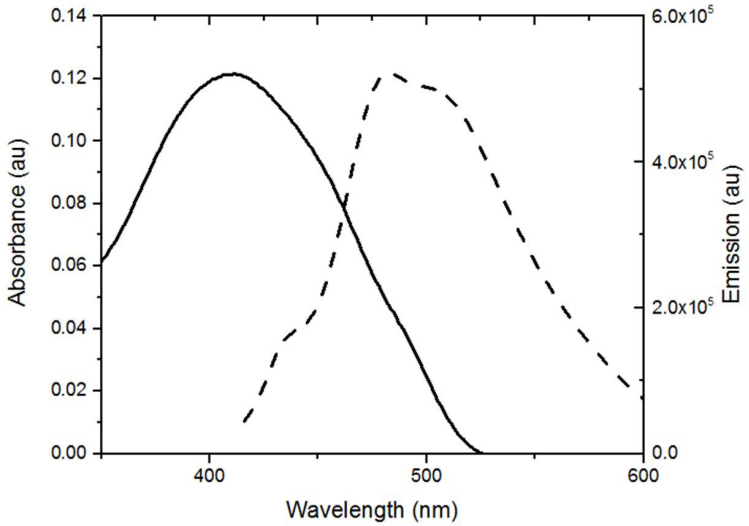


Fig. S-4 Absorption and emission spectra for **CPN-2** in water (Excitation = 400 nm, slit widths = 3 nm, integration time = 0.1 s).

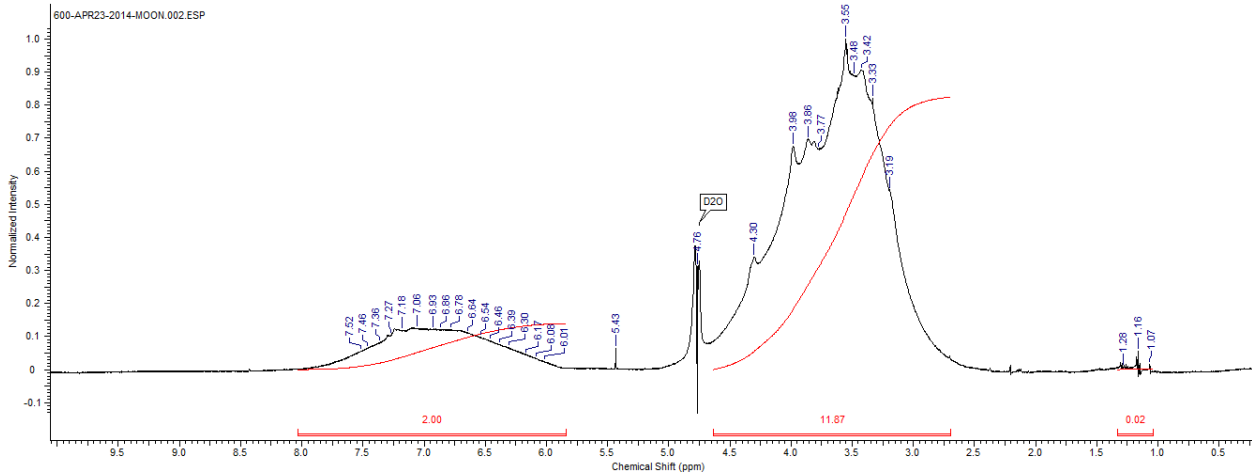


Fig. S-5 ^{1}H NMR (600 MHz) of **CPN-2** in D_2O.

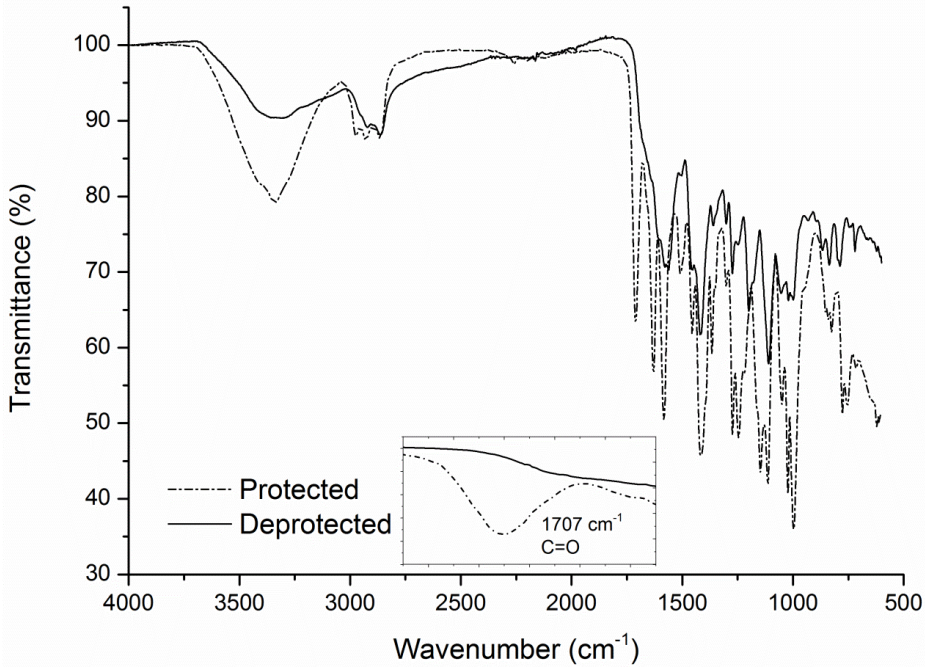


Fig. S-6 FT-IR of **P2** (neat).

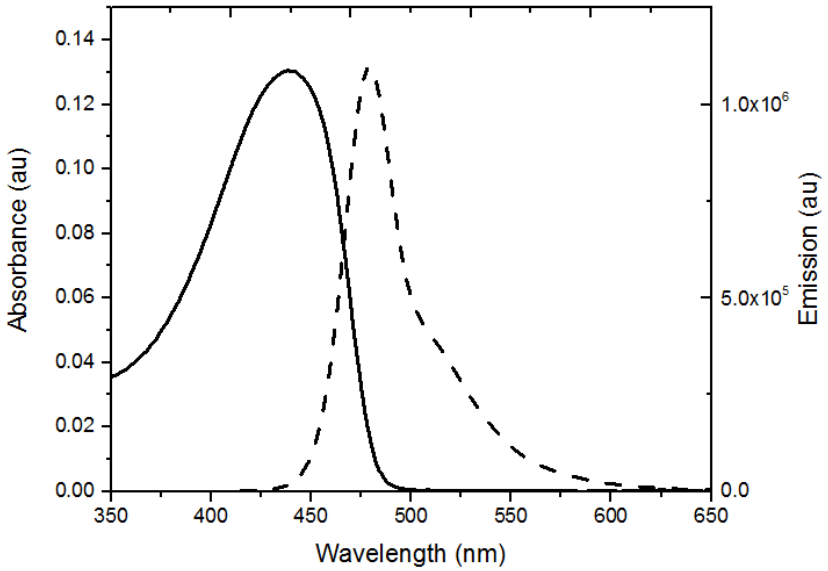


Fig. S-7 Absorption and emission spectra for **P3** in DMF (Excitation = 400 nm, slit widths = 3 nm, integration time = 0.1 s).

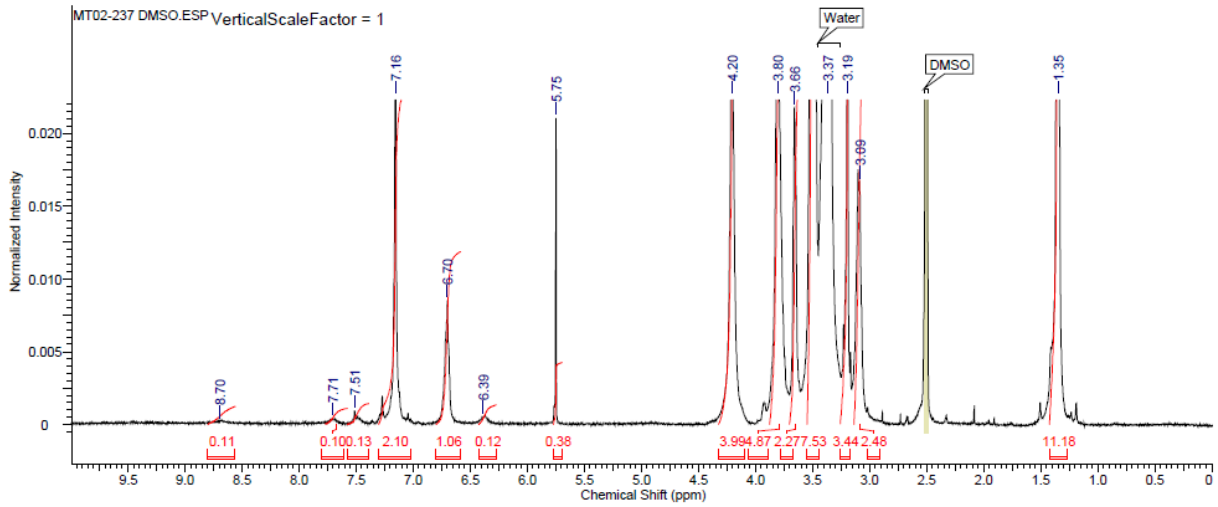


Fig. S-8 ^{1}H NMR (400 MHz) of **P3** in DMSO-d6.

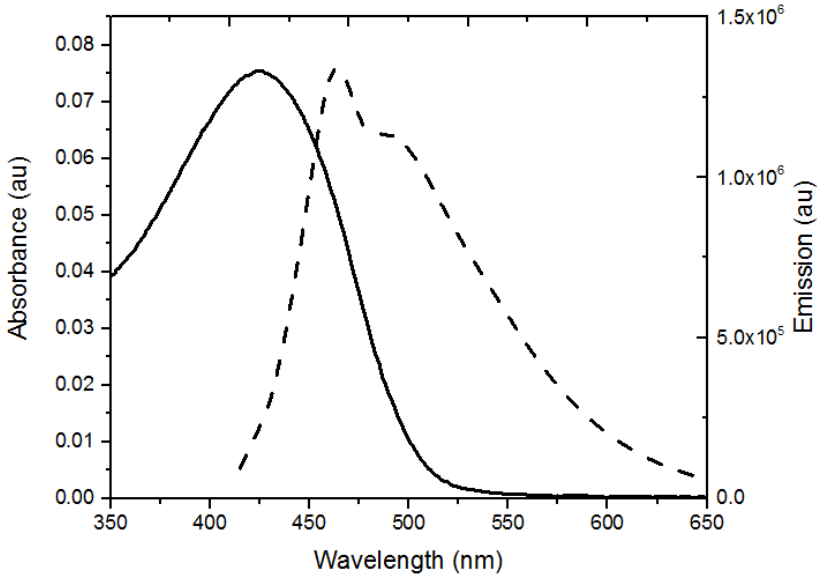


Fig. S-9 Absorption and emission spectra for **CPN-3** in water (Excitation = 400 nm, slit widths = 3 nm, integration time = 0.1 s).

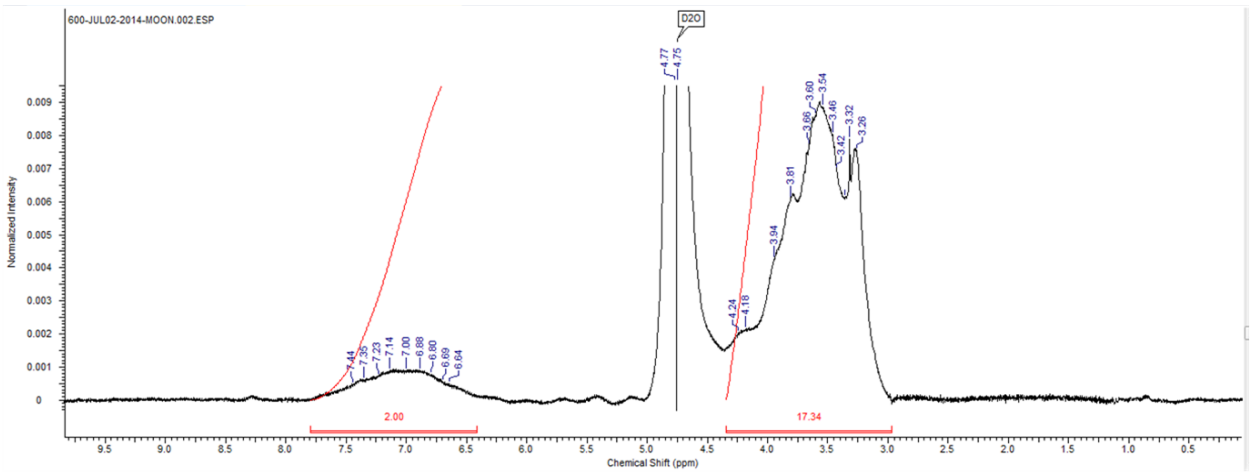


Fig. S-10 ^{1}H NMR (600 MHz) of **CPN-3** in D_2O.

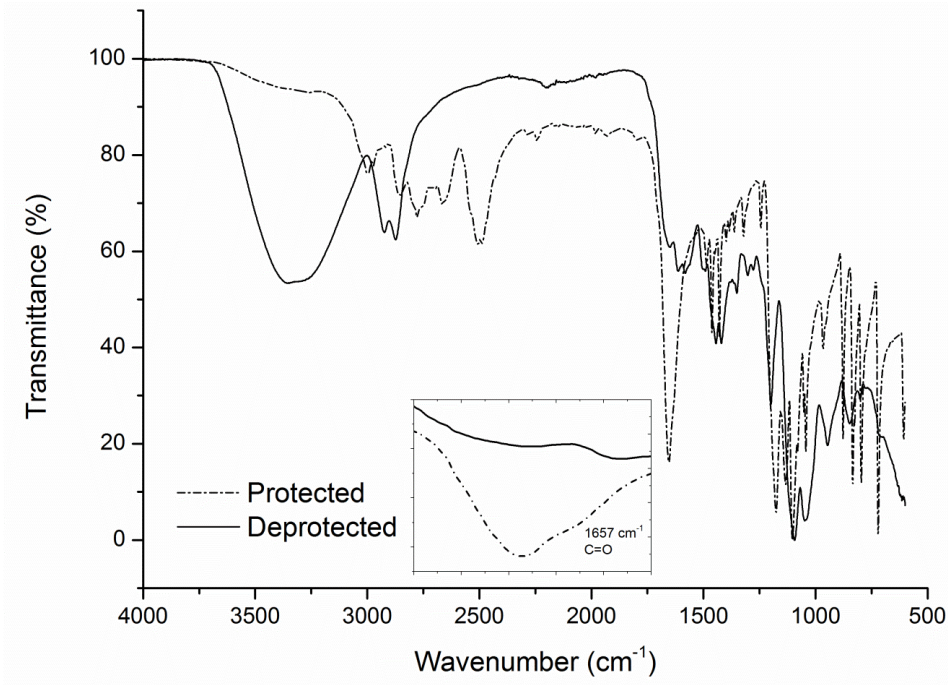


Fig. S-11 FT-IR of **P3** (neat).

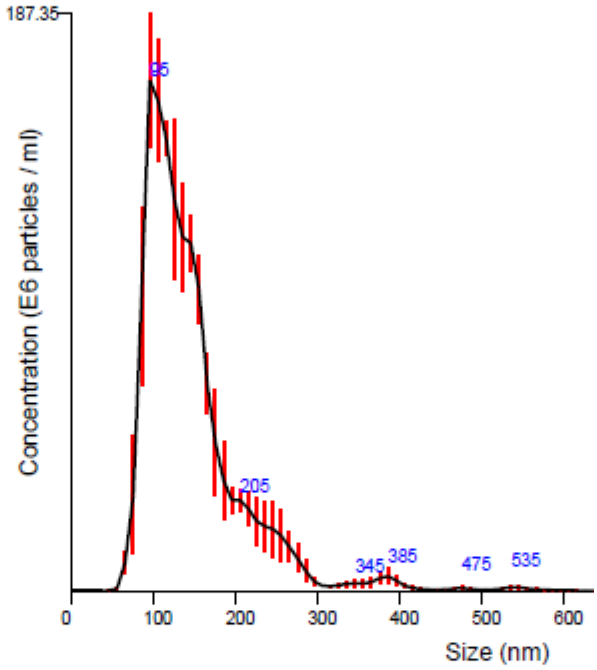


Fig. S-12 NTA of **CPN-1** in water.

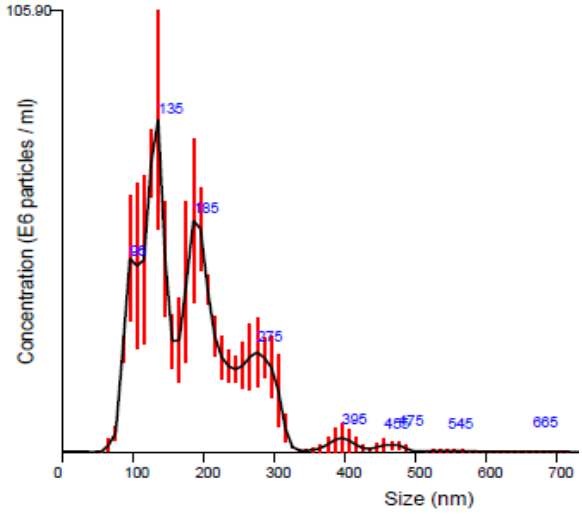


Fig. S-13 NTA of **CPN-2** in water.

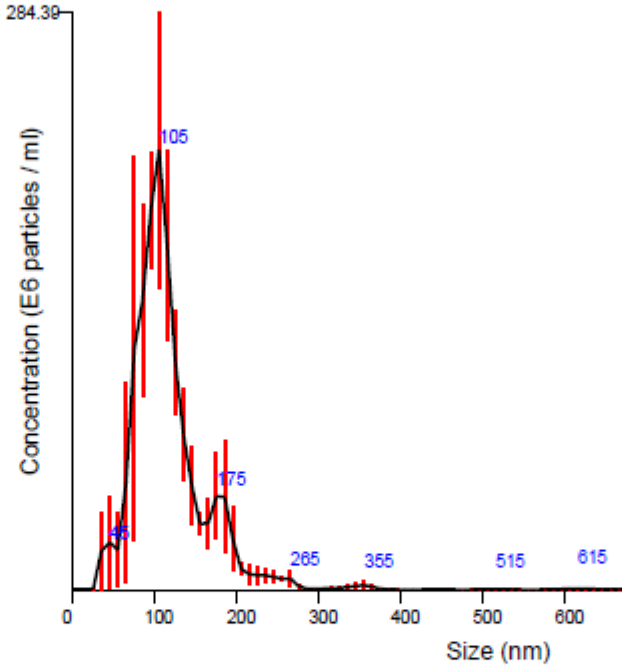


Fig. S-14 NTA analysis of **CPN-3** in water.

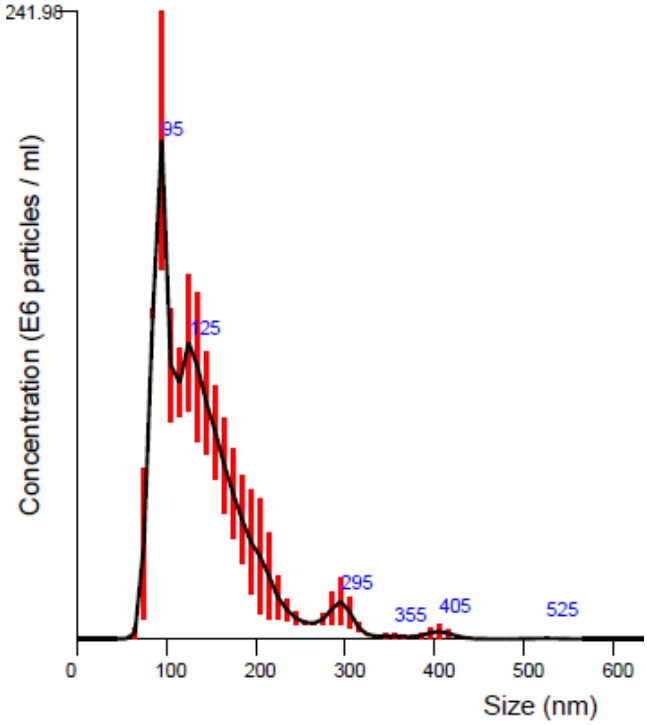


Fig. S-15 NTA of **CPN-4** in water.

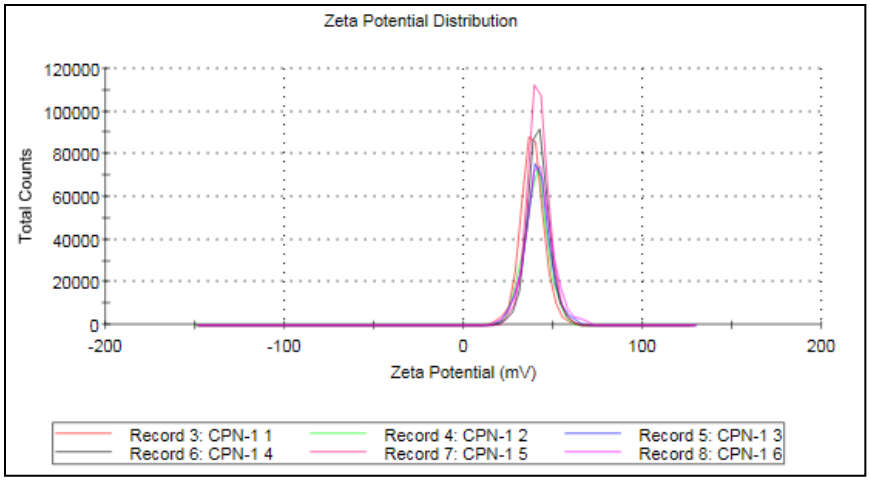


Fig. S-16 Zeta potential of **CPN-1** in water.

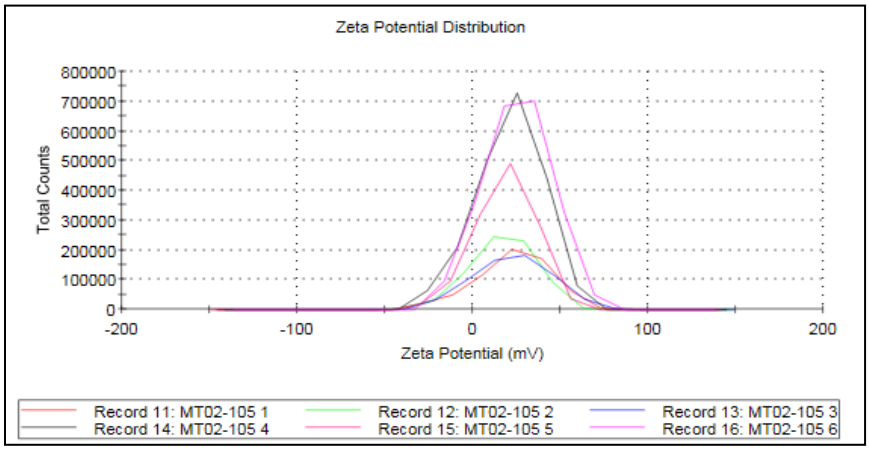


Fig. S-17 Zeta potential of **CPN-2** in water.

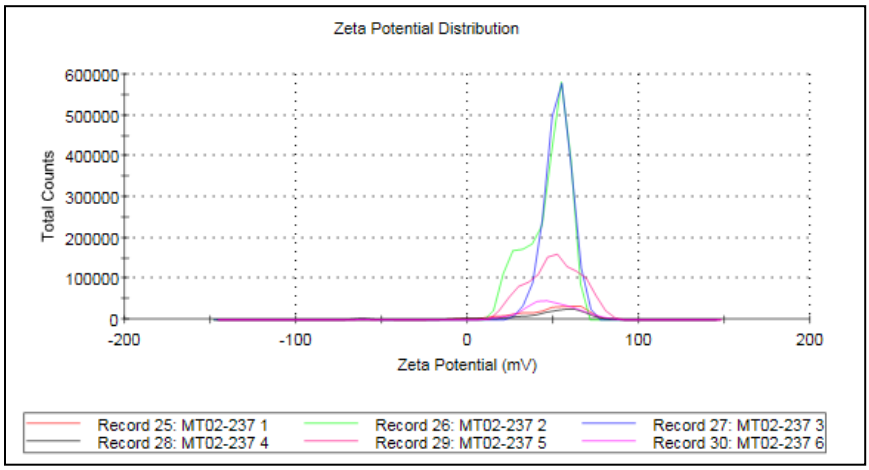


Fig. S-18 Zeta potential of **CPN-3** in water.

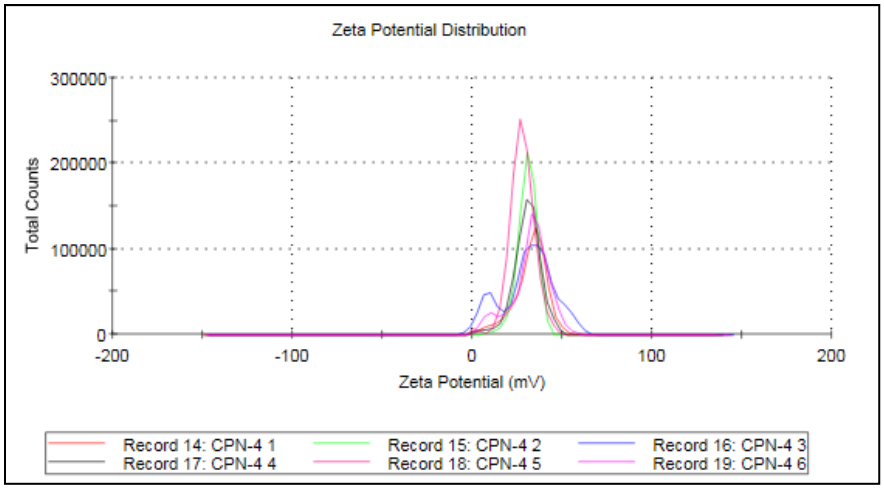


Fig. S-19 Zeta potential of **CPN-4** in water.

Table S-1 Summary of NTA data for CPN/GAG complexes.

Sample	Mean (nm)	Mode (nm)	SD[a] (nm)
CPN-1	140 ± 0.9	108 ± 8.4	63 ± 3.2
+ HA	174 ± 1.4	145 ± 9.4	65 ± 3.9
+ HS	171 ± 1.9	142 ± 7.7	58 ± 1.9
+ CS	177 ± 4.8	148 ± 13.3	82 ± 8.2
+ DS	167 ± 1.6	159 ± 0.6	58 ± 3.2
CPN-2	179 ± 6.3	125 ± 6.5	74 ± 5.8
+ HA	152 ± 2.7	138 ± 8.5	62 ± 5.3
+ HS	162 ± 1.1	124 ± 7.1	70 ± 3.3
+ CS	137 ± 2.1	111 ± 6.0	59 ± 3.7
+ DS	137 ± 2.3	114± 3.2	59 ± 3.7
CPN-3	118 ± 10.1	90 ± 7.8	66 ± 23.5
+ HA	150 ± 4.6	125 ± 4.6	57 ± 4.7
+ HS	151 ± 5.4	125 ± 2.5	45 ± 4.9
+ CS	165 ± 6.4	149 ± 1.6	60 ± 5.1
+ DS	140 ± 1.3	125 ± 4.2	47 ± 3.5
CPN-4	135 ± 8.1	95 ± 8.1	55 ± 3.8
+ HA	170 ± 4.4	132 ± 6.6	75 ± 8.2
+ HS	187 ± 2.7	144 ± 5.3	87 ± 11.8
+ CS	216 ± 2.6	151 ± 3.7	97 ± 5.2
+ DS	216 ± 1.9	148 ± 6.6	92 ± 2.8

[a]SD is the standard deviation characteristic of the width of the mean peak.

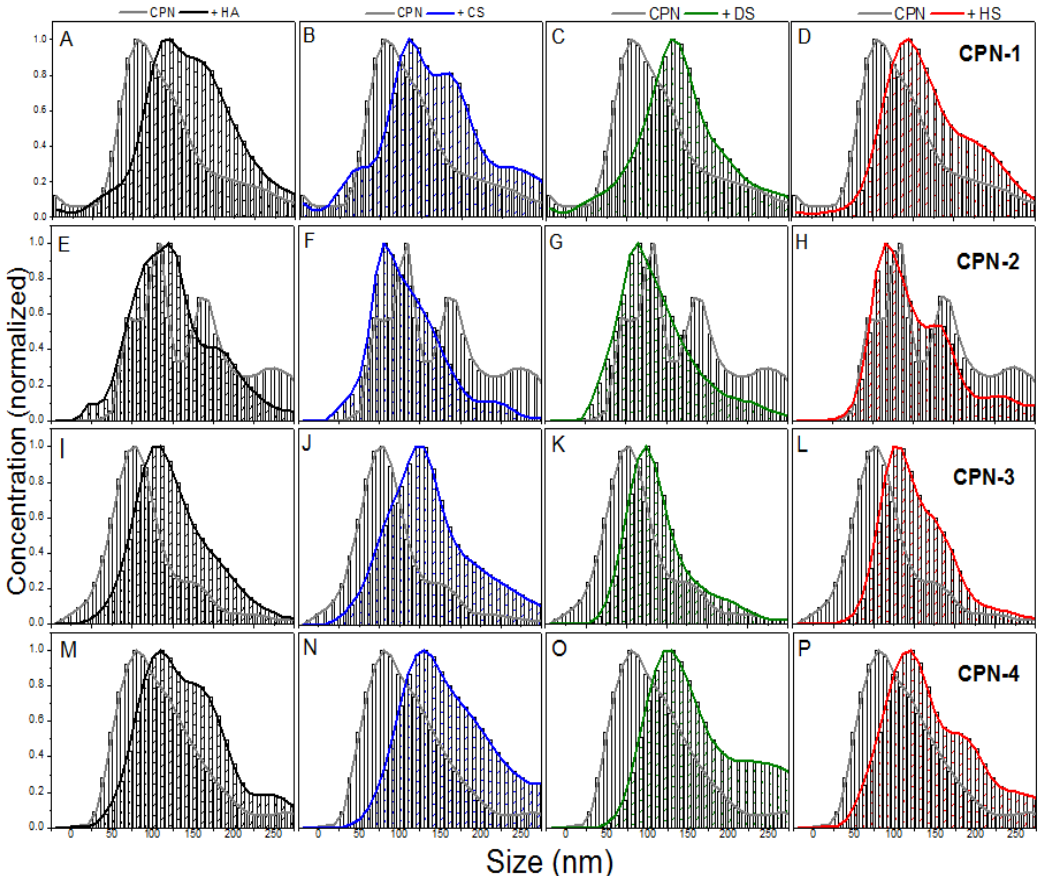


Fig. S-20 Average size distributions of CPNs complexed with GAGs.

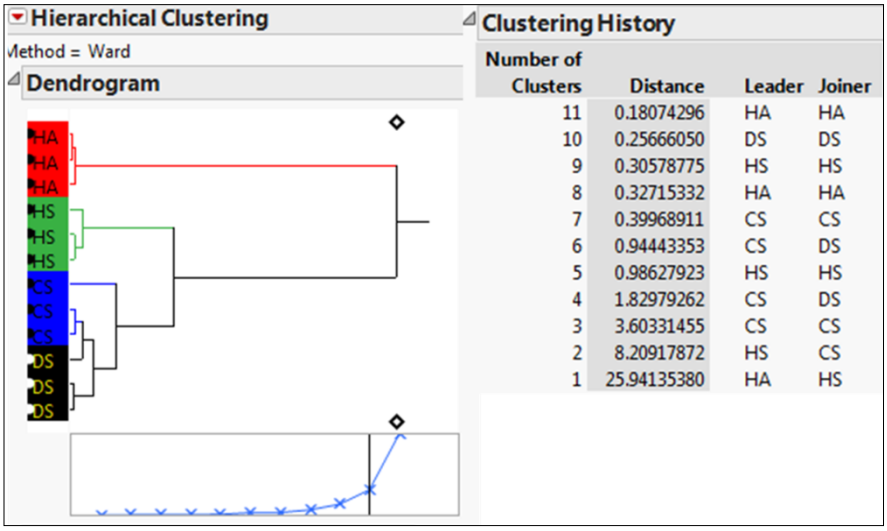


Fig. S-21 Hierarchical cluster analysis (HCA) of CPN-2+GAGs in water.

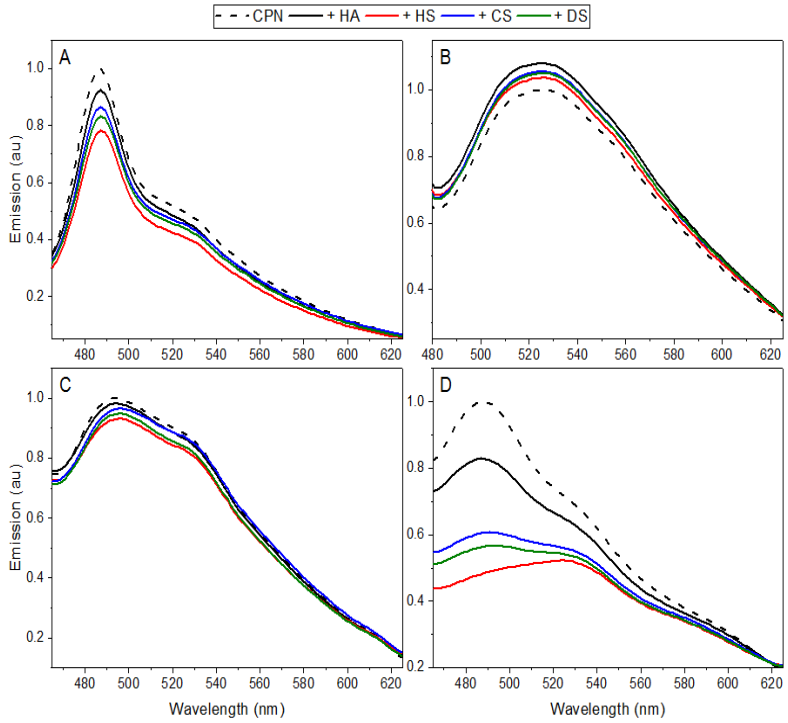


Fig. S-22 Emission spectra of CPN-1 (a), CPN-2 (b), CPN-3 (c), and CPN-4 (d) in the presence of GAG-containing urine simulant. Excitation wavelength for all CPNs was 450 nm with 5 nm slit widths, and 0.5 s integration time.

Table S-2 Summary of canonical scores from LDA plot.

Canonical	Eigenvalue	Percent of variation	Canonical correlation	Likelihood ratio	P-value
1	51.1592	71.0561	0.9903	0.00015	<.0001
2	12.0357	16.7166	0.9608	0.00782	<.0001
3	8.8034	12.2273	0.9476	0.10200	<.0001

Table S-3 Squared Mahalanobis distances to each group centroid.

Row	Actual	CS	DS	HA	HS
1	CS	40.333331755	12294.647468	4063.5247309	7101.4731662
2	CS	40.33333189	12318.702339	4091.8694316	7100.0157848
3	CS	40.333331247	12295.354372	4055.8629135	7077.7114241
4	CS	40.333332814	12320.127561	4063.8690325	7028.8745497
5	CS	40.333332332	12284.219683	4022.5165612	7068.8817155
6	CS	40.33333311	12305.608646	4078.0823462	7070.0813947
7	CS	40.333332299	12324.225381	4101.1928134	7029.6991414
8	CS	40.333332583	12297.892499	4043.5769265	7080.2601624
9	CS	40.333332368	12290.603231	4079.2351324	7099.9786147
10	CS	40.333332042	12317.900963	4084.3114748	7104.2194088
11	CS	40.333332023	12321.138359	4086.9472166	7088.7210233
12	CS	40.333331329	12327.718608	4068.9691099	7091.0784818

13	DS	12336.168794	40.333332198	4485.4027952	5017.6750565
14	DS	12308.32746	40.333331405	4461.5478914	5024.6443335
15	DS	12285.66102	40.333332437	4444.505515	4960.6845733
16	DS	12322.927181	40.333333024	4494.3974088	5092.3506657
17	DS	12284.738557	40.33333405	4431.2023819	5018.2556851
18	DS	12309.48622	40.333333605	4465.5395729	4968.2806311
19	DS	12308.350358	40.333333771	4497.3940618	4981.3115882
20	DS	12299.854928	40.333333821	4458.0858604	5006.5912977
21	DS	12288.977123	40.333333818	4449.3440504	5029.0243225
22	DS	12316.660014	40.333333525	4479.2810227	5006.1744725
23	DS	12309.957045	40.333332342	4471.8327096	5016.2428069
24	DS	12327.030404	40.333332195	4490.8880232	5031.904691
25	HA	4071.5727552	4479.4540603	40.333334415	4189.6090669
26	HA	4051.7432194	4464.1781327	40.333333094	4181.7118782
27	HA	4038.7851613	4447.7852168	40.333333772	4226.4907325
28	HA	4069.2033941	4452.9086196	40.333333224	4166.0945872
29	HA	4043.7940084	4465.4116832	40.33333387	4101.2929977
30	HA	4091.1485334	4485.9916447	40.333333783	4207.6709894
31	HA	4087.8395223	4435.7896652	40.333333211	4216.5914613
32	HA	4079.9438813	4435.5311514	40.333333177	4219.9600424
33	HA	4067.9651299	4520.1123035	40.333333286	4148.2627038
34	HA	4091.700492	4486.0424938	40.333333274	4184.9552956
35	HA	4093.2678457	4481.8711676	40.333333602	4202.0467871
36	HA	4052.9937363	4474.345146	40.333333464	4153.3574859
37	HS	7096.5304652	5022.950778	4191.1591296	40.333333658
38	HS	7074.0622865	5010.9473948	4206.4649295	40.333333755
39	HS	7059.2479562	4991.6573071	4181.3105117	40.333334356
40	HS	7096.8954728	5039.1040348	4194.8850944	40.333334461
41	HS	7070.6823557	4973.1371849	4165.069548	40.33333396
42	HS	7087.2351087	5032.825679	4206.7409419	40.333333788
43	HS	7056.5975622	4986.0591011	4134.249703	40.33333355
44	HS	7081.3330154	5052.2305167	4202.4199089	40.3333335
45	HS	7060.9924572	4989.4852004	4150.13598	40.333333518
46	HS	7083.6742369	5025.4448787	4194.4434217	40.333333149
47	HS	7095.8214509	5018.3121938	4188.5907513	40.333333749
48	HS	7077.9224838	5010.9858432	4182.5741073	40.333332594

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