Supporting information

Polymer Mediated Tuning of Monomer-Aggregate Equilibrium of A Coumarin Derivative for Ratiometric Sensing of Protamine

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S.N.	Table of Content	Page
1	Experimental section.	S3-S4
2	**Fig. S1.** Normalized absorption spectra of C7 (30 μM) in 2 mM citrate buffer (pH ~3) at (1) 0 and (10) 87.1 nM PSS.	S5
3	**Fig. S2. (A)** Modulations in emission spectra of C7–PSS complex (30 μM C7+85 nM PSS) in water with change in pH of the solution from 7.30 to 12.92. **(B)** Ratiometric change in emission intensity for the complex from pH 2.0 to 12.92.	S5
4	**Fig. S3.** Size distribution obtained from atomic force microscopy for C7 aggregates formed under the influence of PSS.	S6
5	**Fig. S4.** AFM images of **(A)** 30 μM C7, **(B)** 85 nM PSS and **(C)** C7-PSS complex (30 μM C7+85 nM PSS).	S6-S7
6	**Fig. S5.** Particle size distribution of 30 μM C7 and C7-PSS (30 μM C7+85 nM PSS) in 2 mM phosphate buffer (pH 7.0) and 2 mM bicarbonate-carbonate buffer, (pH 9.2).	S7
7	**Fig. S6.** Peak normalized absorption spectra of C7-PSS complex (30 μM C7+85 nM PSS) in 2 mM citrate buffer (pH ~3) at various concentrations of NaCl (indicated in the inset).	S8
8	**Fig. S7.** Excited-state decay profiles of C7-PSS complex (30 μM C7+85 nM PSS) with increasing concentration of NaCl in 2 mM citrate buffer (pH ~3).	S8
9	**Fig. S8.** Linear fit of the ratiometric changes of peak emissions for the C7–PSS complex with Pr concentration in the range of 0 to 0.2 μM for LOD determination.	S9

Experimental Section

1. Materials and methods of sample preparation

Laser grade Coumarin 7 [3-(2-Benzimidazolyl)-7-(diethylamino)coumarin] was procured from Exciton, UK and used without any further purification. Polystyrene sulfonate (PSS, average molecular weight ~70,000) and protamine sulfate (Grade X) utilized in this study were procured from Sigma-Aldrich and used as obtained. All inorganic salts (NaCl, KCl, $MgCl_2$, $CaCl_2$, $ZnCl_2$, $CuSO_4$, $FeSO_4$, and $Al(NO_3)_3$), bio-analytes [amino acids, adenosine triphosphate disodium salt (ATP), alpha amylase, bovine serum albumin (BSA), bovine beta lactoglobulin (BLG), carbonic anhydrase from bovine erythrocytes, CTAB, glucose, lipase, lysozyme from egg white, pepsin, protease, and trypsin], biogenic amines (cadaverine, putrescine, spermidine, spermine) were purchased from Sisco research laboratories (SRL), India and utilized as received. Since C7 is insoluble in water, concentrated ethanolic stock of C7 was diluted with appropriated amount of water to make final concentration of aqueous C7 ~30 μM for the study.

Complexation of C7 with PSS is greatly affected by pH of the medium but in the pH range of 2.0 to 4.5 formation of C7-PSS complex remains almost unaltered (discussed in the main manuscript), we thus, performed our study in 2 mM citrate buffer of pH ~3. In the pH dependent emission study, pH of the sample solution was altered using diluted NaOH and HCl solutions.

2. Photophysical studies

A double beam UV-Vis spectrophotometer (Model No: UV-2700 of Shimadzu, Japan) was used to record ground-state absorption spectra of the sample in a quartz cuvette of 1 cm x 1 cm path length. The steady-state (SS) emission spectra of the samples were obtained using a spectrofluorimeter (Model no: Fluoromax-4 of Horiba, UK) in the same cuvette. The sample solutions in SS emission studies were excited at ~445 nm to avoid any artifact due to the change in optical density during complexation process. A time-correlated single photon counting (TCSPC) spectrometer (Model no: FLS-980 of Edinburgh instrument, U.K) was utilized for obtaining the excited-state lifetime of the samples. A 405 nm pulsed diode laser (EPL-405, pulse width ~62 ps and pulse repetition 10 MHz) was used to excite the samples and the decay traces were recorded at 513 nm and 650 nm using a detection module based on photomultiplier tube (PMT). To avoid any contribution arising due to anisotropy in the decay traces, a magic angle (54.7 ^oC) configuration was always maintained during recording of the excited-state decay traces

for the samples. The lamp profile, also known as instrument response function (IRF or prompt) was recorded to be ~250 ps at FWHM from the scattered light of aqueous suspension of ludox. Fluorescence decay traces were fitted using a reconvolution analysis, using a poly-exponential decay function,[1]

$$I(t) = I(0)\sum \alpha_i \exp(-t/\tau_i) \quad (1)$$

Where, *I(0)* is the initial intensity at time, t = 0; whereas α_i and τ_i are the relative pre-exponential factor and fluorescence lifetime, respectively, for the i^{th} decay component.

Average lifetimes for the excited-state decays were calculated using equation,[1]

$$<\tau>=\sum A_i \tau_i \text{ where, } \quad A_i = \alpha_i \tau_i / \sum \alpha_i \tau_i \quad (2)$$

All the photophysical studies were performed at ambient temperature i.e., at ~25 ^{o}C, otherwise mentioned explicitly.

3. Atomic force microscopy (AFM)

AFM image for C7, PSS and C7-PSS complex were obtained using Atomic Force Microscope (NTMDT Ntegra). On sapphire substrate adhered mica surface, ~10 µL of freshly prepared C7 (30 µM), PSS (85 nM) and C7-PSS complex (mixture of 30 µM C7 and 85 nM PSS) in 2 mM citrate buffer (pH ~3) were spotted individually. The samples were then dried carefully and slowly under infra-red lamp for approximately 30 minutes. After that the samples (mica plates) were scanned with 100-micron scanner attached to a SMENA head with NSG11 golden silicon probes using semi-contact topography mode. NTMDT NOVA 1.1.01780 software was used for the measurement of the height of 50 randomly selected samples using arbitrary height measurement tool discussed somewhere else.[2]

4. Dynamic light scattering (DLS)

Litesizer™ 500 from Anton Paar (United States), containing a diode laser of 658 nm wavelength and 40 mW power, was used for obtaining the DLS measurements at a back-scattering angle of 175^{o} using a photomultiplier tube detector. The measurements were replicated for at least three times to get the average size of the particles. The hydrodynamic diameter (d_h) for the particles was obtained using the Stokes-Einstein equation, $D = k_B T/3\pi\eta d_h$, where, k_B = Boltzman Constant, T= absolute temperature, D= diffusion coefficient, η = viscosity of solvent.

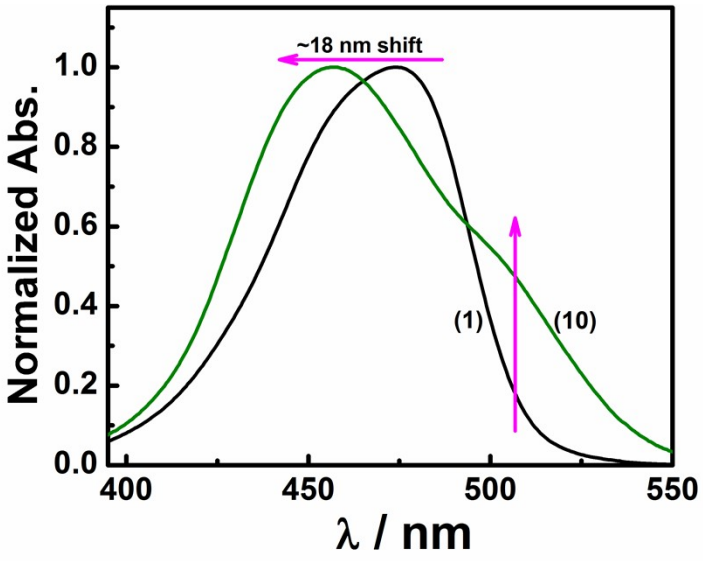

Fig. S1. Normalized absorption spectra of C7 (30 μM) in 2 mM citrate buffer (pH ~3) at (1) 0 and (10) 87.1 nM PSS.

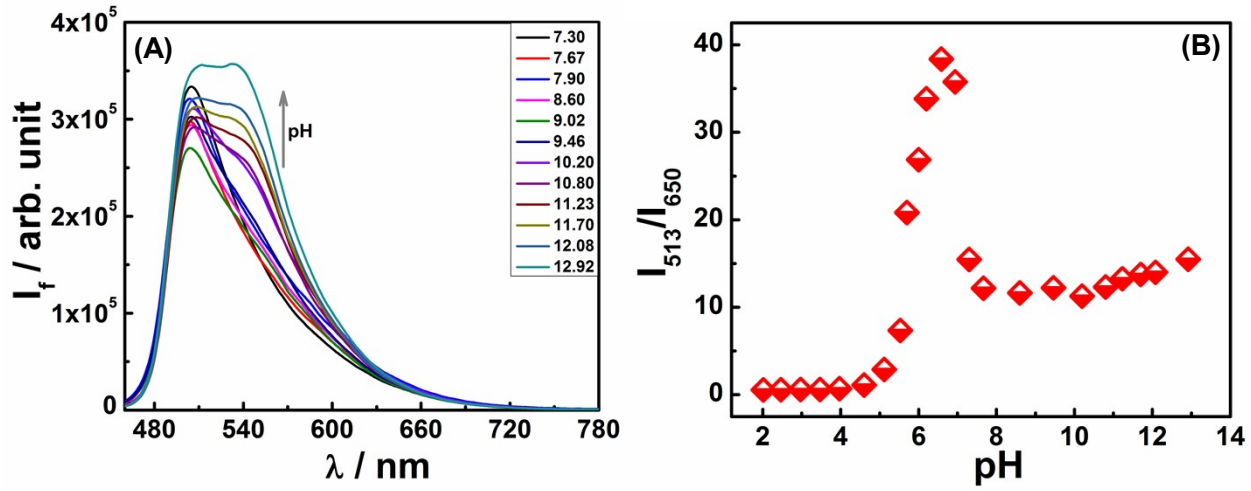

Fig. S2. (A) Modulations in emission spectra of C7–PSS complex (30 μM C7+85 nM PSS) in water with change in pH of the solution from 7.30 to 12.92. **(B)** Ratiometric change in emission intensity for the complex from pH 2.0 to 12.92. λ_{ex} = 445 nm.

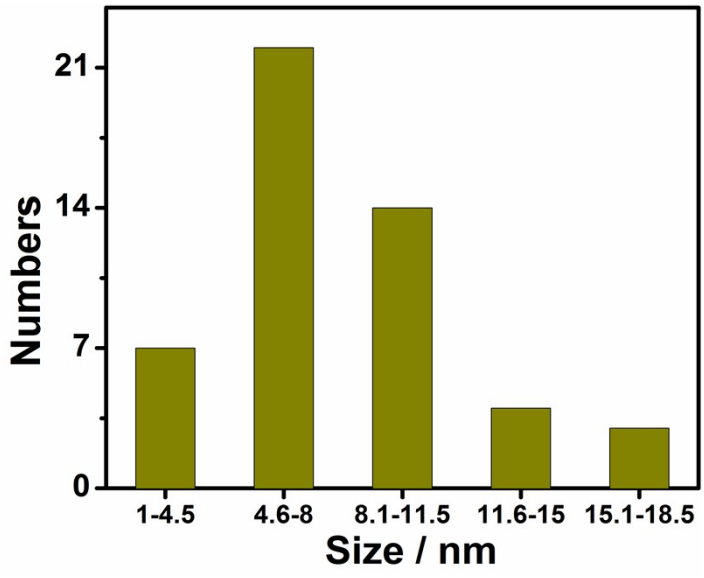

Fig. S3. Size distribution obtained from atomic force microscopy for C7 aggregates formed under the influence of PSS.

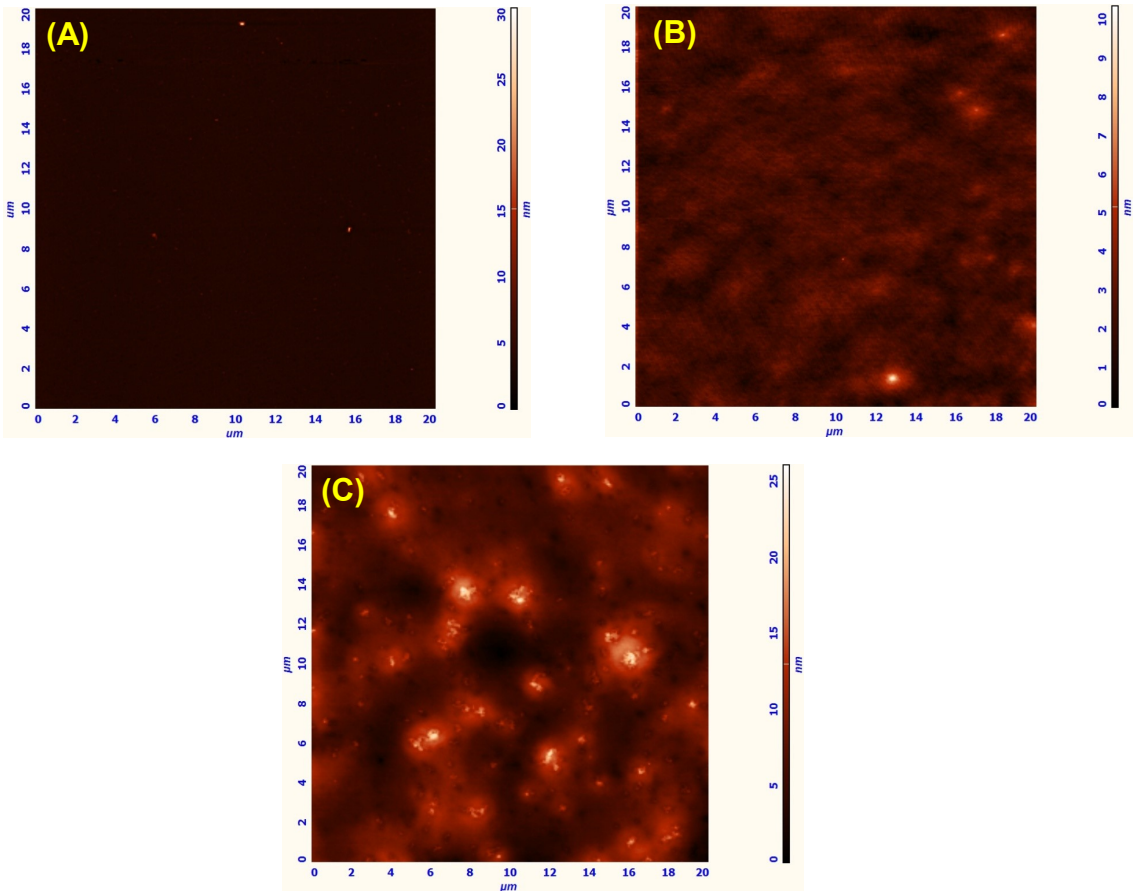

Fig. S4. AFM images of **(A)** 30 μM C7, **(B)** 85 nM PSS and **(C)** C7-PSS complex (30 μM C7+85 nM PSS).

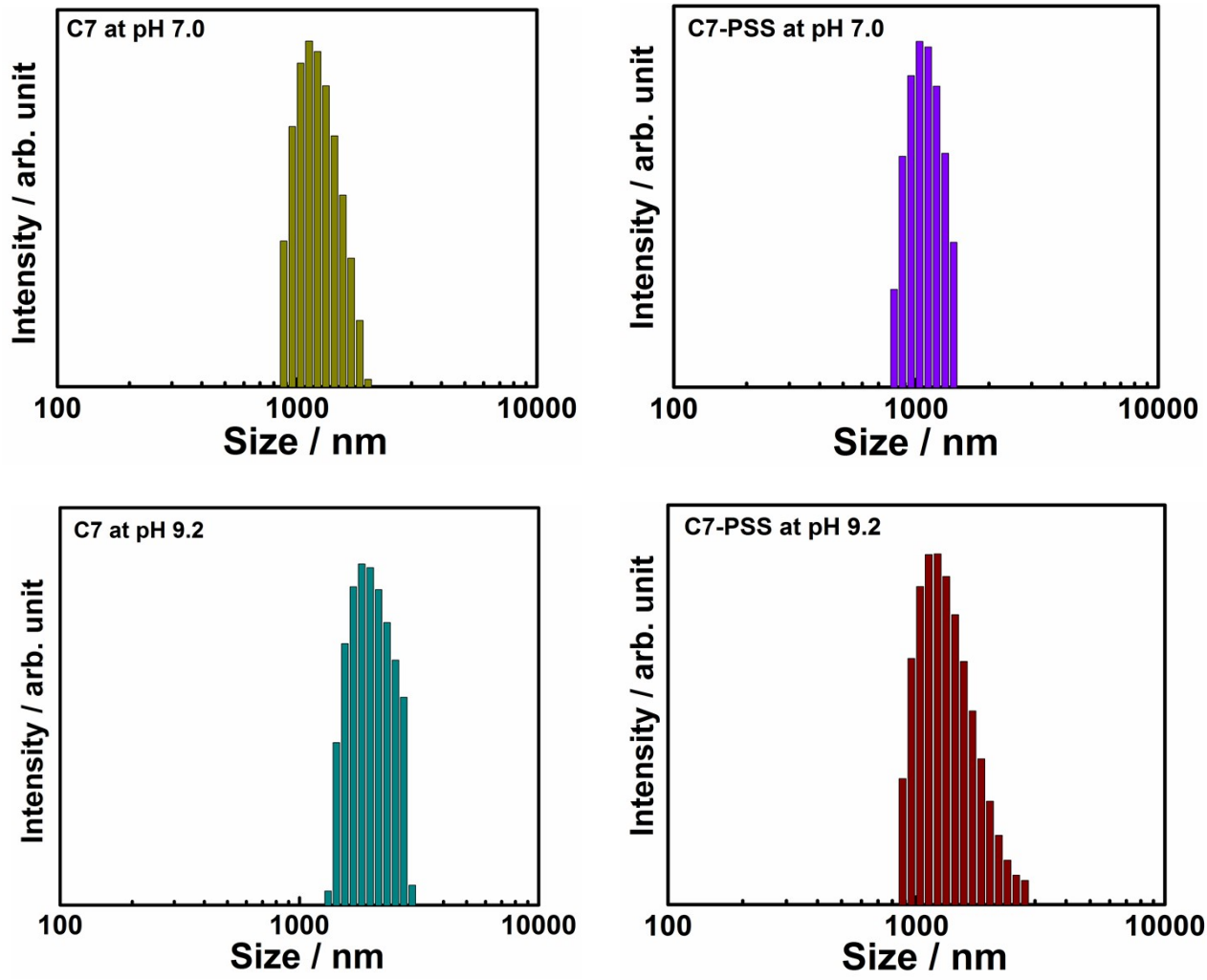

Fig. S5. Particle size distribution of 30 μM C7 and C7-PSS (30 μM C7+85 nM PSS) in 2 mM phosphate buffer (pH 7.0) and 2 mM bicarbonate-carbonate buffer (pH 9.2).

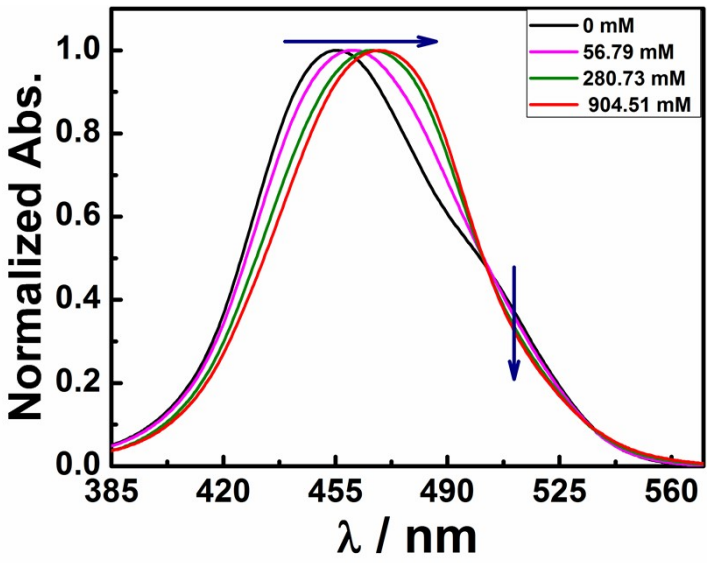

Fig. S6. Peak normalized absorption spectra of C7-PSS complex (30 μM C7+85 nM PSS) in 2 mM citrate buffer (pH ~3) at various concentrations of NaCl (indicated in the inset).

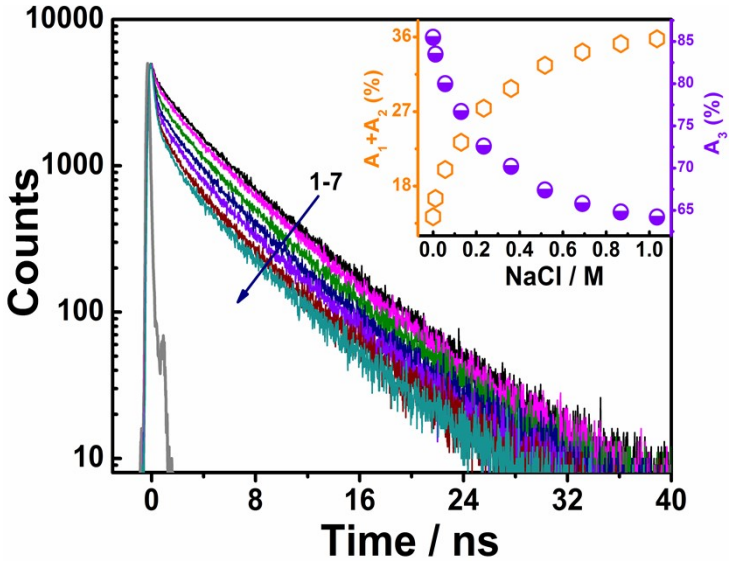

Fig. S7. Excited-state decay profiles of C7-PSS complex (30 μM C7+85 nM PSS) with increasing concentration of NaCl in 2 mM citrate buffer (pH ~3). For profile 1 to 7 concentrations of NaCl are (in mM): 0, 11.4, 55.9, 129.4, 234.5, 517.5, and 1035.0. Inset: Increase in contribution of monomeric species (A_1+A_2) at the expense of aggregate (A_3) with incremental addition of NaCl. λ_{ex} = 405 nm and λ_{em} = 650 nm.

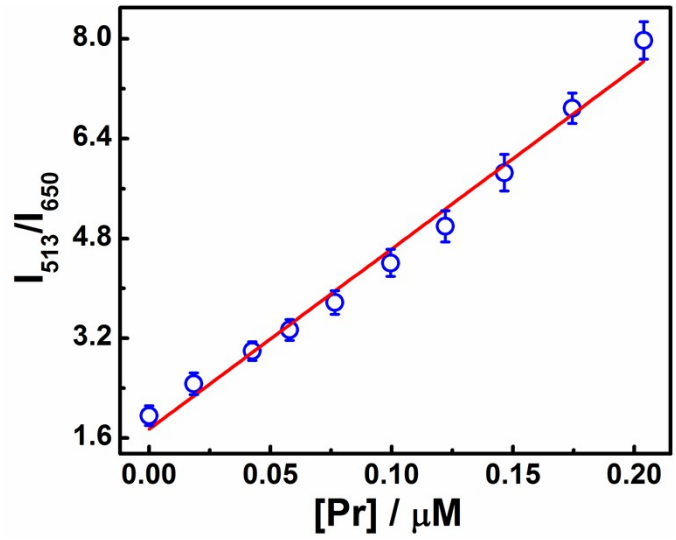

Fig. S8. Linear fit of the ratiometric changes of peak emissions for the C7–PSS complex with Pr concentration in the range of 0 to 0.20 μM for LOD determination. $I_{513/650}$ = 2.89 x 10 [Pr/μM] +1.74, R^2 = 0.987, standard dev (σ) = 0.027 and LOD ~2.8 nM.

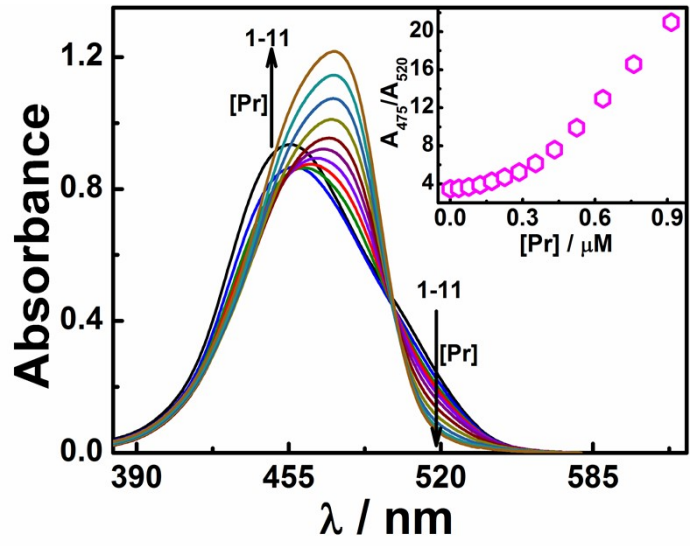

Fig. S9. Changes in absorption spectra of C7-PSS complex (30 μM C7+85 nM PSS) in 2 mM citrate buffer (pH ~3) at different concentrations of protamine. For spectra 1 to 12 concentrations of Pr are: 0, 77.1, 172.7, 226.2, 285.5, 353.5, 432.3, 525.1, 632.4, 760.5 and 916.2 nM. Inset: Ratiometric change in peak absorptions for the C7-PSS complex with increasing Pr concentration.

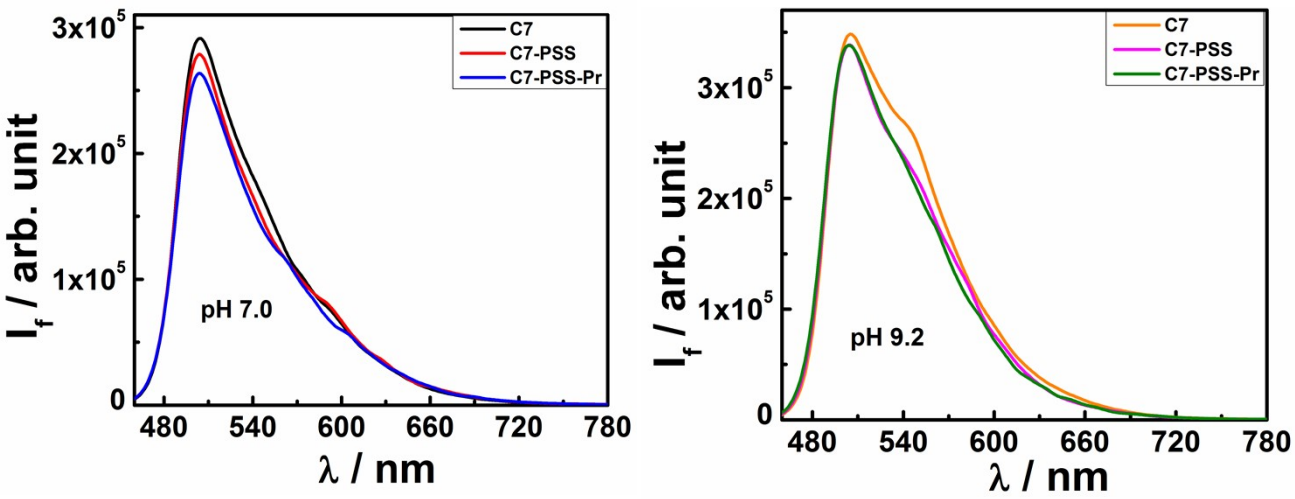

Fig. S10. Emission spectra of C7, C7-PSS complex (30 μM C7+85 nM PSS) and C7-PSS-Pr in 2 mM phosphate and bicarbonate-carbonate buffer. λ_{ex} = 445 nm

<u>Explanation</u>: We also explored the possibility of protamine sensing at pH 7 and pH 9.2. Interestingly, we found that at both pH 7 and 9.2, free dye, dye-PSS and dye-PSS-Pr did not show any difference in fluorescence property. This clearly indicates that at both the pH conditions, the dye C7 does not interact with PSS to form C7-PSS complex mainly due to the absence of dominant electrostatic interaction between neutral C7 and anionic PSS. For this reason protamine hardly causes any modulation to the C7-PSS solution. This further justifies the use of pH 3 for the sensing of protamine using C7-PSS complex.

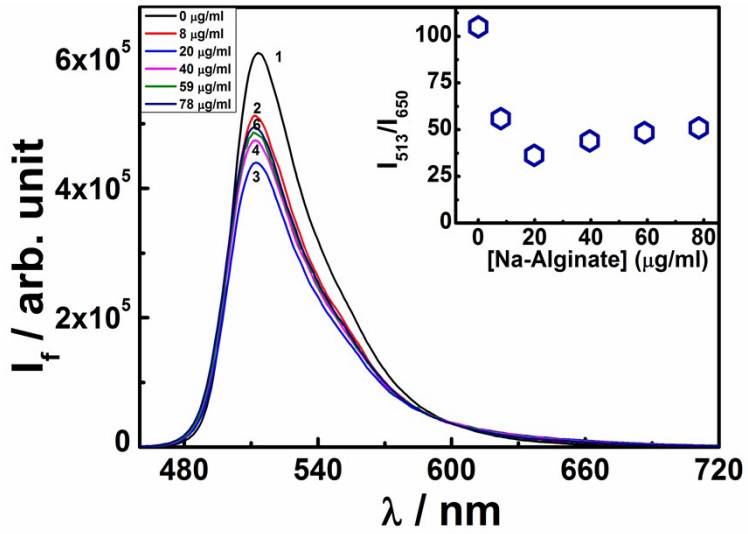

Fig. S11. Emission spectra of C7 (30 μM) with increasing Na-alginate concentration in 2 mM phosphate buffer (pH ~3), λ_{ex} = 445 nm. Inset: Ratiometric change in peak emission for the C7 with increasing Na-alginate concentration.

Explanation: We used another anionic polymer (sodium alginate) to study the aggregation process and result has been depicted in Fig. S10, ESI. Although initially there is decrease in emission peak of the dye with increasing Na-alginate concentration, indicating aggregation of C7 but beyond 20 μg/ml of the polymer concentration hardly any change in the emission feature for the dye was observed. This is clearly supports the use of PSS for C7 aggregation due to the strong electrostatic interaction as well as hydrophobic interaction offered by PSS towards C7.

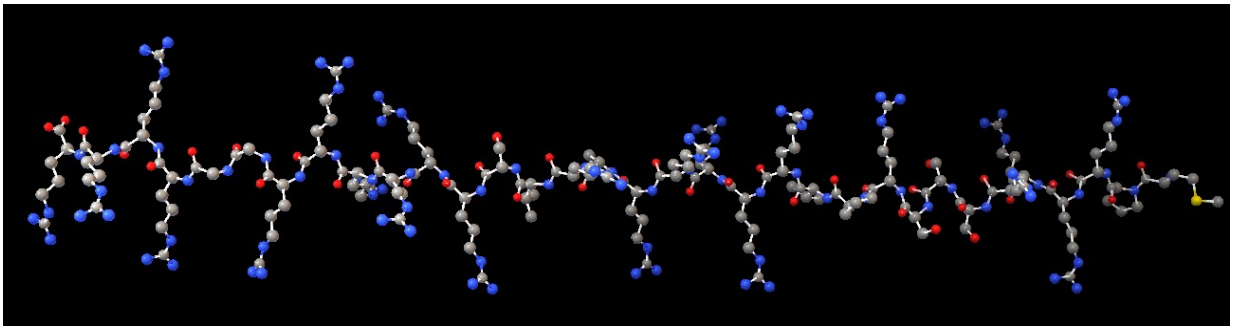

Fig. S12. PDB (P69014) file of protamine obtained from AlphaFold Protein structure database. (Blue : Nitrogen atoms, grey: Carbon atoms, red: oxygen atoms, yellow: Sulfur atom).

Table S1. Excited-state decay parameters of C7–PSS complex (30 μM C7+85 nM PSS) at different NaCl concentrations. λ_{ex} = 405 nm, λ_{em} = 650 nm and pH ~3 (2 mM citrate buffer).

Conc. of NaCl (mM)	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	τ_3 (ns)	A_3 (%)	τ_{avg} (ns)
0	0.23	2.1	1.22	12.2	5.59	85.5	4.93
11.4	0.23	3.3	1.22	13.2	5.52	83.5	4.78
55.9	0.23	5.8	1.22	14.2	5.44	80.0	4.54
129.	0.23	8.9	1.22	14.4	5.33	76.7	4.28
234.5	0.23	11.7	1.22	15.7	5.31	72.6	4.07
360.8	0.23	14.0	1.22	15.8	5.29	70.2	3.94

517.5	0.23	15.8	1.22	16.8	5.26	67.4	3.79
690.0	0.23	17.4	1.22	16.8	5.25	65.8	3.70
868.1	0.23	18.4	1.22	16.8	5.20	64.8	3.62
1035	0.23	18.3	1.22	17.5	5.20	64.2	3.59

Table S2. Excited-state decay parameters of C7–PSS complex (30 μM C7+85 nM PSS) at different Pr concentrations. λ_{ex} = 405 nm, λ_{em} = 650 nm and pH ~3 (2 mM citrate buffer).

Conc. of Pr (μM)	**τ_1 (ns)**	**A_1 (%)**	**τ_2 (ns)**	**A_2 (%)**	**τ_3 (ns)**	**A_3 (%)**	**τ_{avg} (ns)**
0	0.23	1.4	1.22	11.1	5.89	87.5	5.29
0.06	0.23	3.0	1.22	12.4	5.76	84.6	5.03
0.12	0.23	5.9	1.22	14.3	5.71	79.8	4.74
0.22	0.23	13.0	1.22	17.0	5.70	70.0	4.22
0.32	0.23	23.3	1.22	18.1	5.65	58.6	3.58
0.42	0.24	37.5	1.22	18.4	5.60	44.1	2.78
0.52	0.23	50.5	1.22	20.5	5.55	29.0	1.97
0.63	0.23	60.0	1.22	22.3	5.50	17.7	1.38
0.76	0.23	63.7	1.22	24.3	5.48	12.0	1.10
0.91	0.23	65.4	1.22	25.2	5.42	9.4	0.96
1.12	0.22	66.7	1.22	25.4	5.40	7.9	0.88

References

1. J. R. Lakowicz, *Principles of fluorescence spectroscopy, 3rd Edition*, Plenumm Press, Springer, New York, 2006.
2. B. B. Karakoçak, J. Liang, S. Kavadiya, M. Y. Berezin, P. Biswas and N. Ravi, *ACS Appl. Nano Mater.*, 2018, **1**, 3682–3692.