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Heparin Triggered Dose Dependent Multi-Color Emission Switching in Water: A Convenient Protocol for Heparinase I Estimation in Real-Life Biological Fluids

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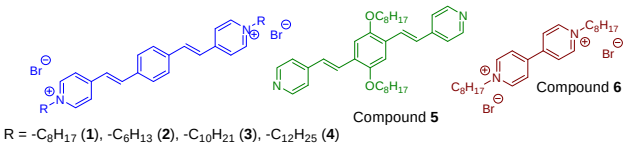
Oligo(*p*-phenylenevinylene) based bis-pyridinium derivatives show 'ratiometric' detection of heparin in water. For the first time, we present a dose-dependent, multi-color emission switching in presence of heparin. Reversible self-assembly of the probes with heparin as the stimulus is also exploited for the screening of heparinase I enzyme.

Heparin is a naturally occurring, highly sulfated linear glycosaminoglycan (GAG) consisting of alternate repeating units of 1-4-linked pyranosyl uronic acid and 2-amino-2-deoxyglucopyranose residues.[1] It can interact with diverse proteins such as antithrombin, fibroblast growth factor, extracellular superoxide dismutase, lipoprotein, *etc.* through electrostatic interactions and thus regulates different physiological and pathological processes such as cell growth and differentiation, inflammation, blood coagulation, lipid transport and metabolism *etc*.[2] Though heparin has been commonly used as an anticoagulant in cardiopulmonary surgery, an increase in heparin level can also result into some adverse effects such as hemorrhages and thrombocytopenia *etc*.[3] Therefore, continuous monitoring and quantification of heparin level in blood serum is crucial for clinical applications during surgery and the post-operative therapy period.

The traditional laboratory assays for heparin estimation are indirect, which mostly rely on monitoring the activated coagulation time (ACT), anti-Xa and the activated partial thromboplastin time (aPTT).[4] However, these methods are expensive and less specific due to potential interference from other biological factors. Therefore, utilization of small organic molecules for specific detection or quantification of heparin has been considered by different research groups. Followed by the pioneering work of Anslyn *et al.* in 2002, a series of colorimetric probes have been developed in the last few years for the recognition of heparin.[5] However, as most of them rendered changes in optical signal at single-point wavelength, the response often found to be influenced by external factors such as, instrumental calibration, variations in light intensity *etc.* In this context, ratiometric fluorescent sensors are superior to others as they can effectively eliminate most or all the interferences from environment by considering the emission changes at two spectral bands (from both unreacted/monomeric and reacted/aggregated probes). Surprisingly, there are very few reports avaliable in literature on ratiometric detection of heparin in water.[6]

Considering these above mentioned challenges, herein, for the first time, we report a dose dependent, heparin induced ratiometric emission switching by controlling the extent of hetero-assembly formation of conjugated bis-pyridinium phenylenevinylene (PPV) based fluorogenic probes in water (Chart 1a). Heparin triggered reversible aggregation phenomenon was further utilized to develop a new technique for heparinase I assay.



R = -C_8H_{17} (**1**), -C_6H_{13} (**2**), -$C_{10}H_{21}$ (**3**), -$C_{12}H_{25}$ (**4**)

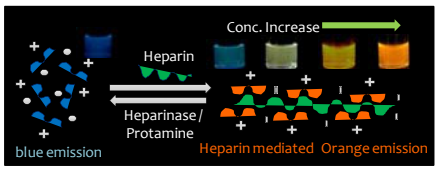


Chart 1. (a) Structures of compounds (**1-6**) used in the present study. (b) Principle for reversible detection of Heparin.

The probe compounds **1-6** have been synthesized following the literature reported procedure (see ESI†). Aqueous solution of the probe **1** (10 μM) exhibited absorption maximum at ~395

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nm along with a strong blue 'monomeric emission' at 470 nm (λ_{ex} = 380 nm) (Fig S1, ESI†). The crucial balance between the hydrophobic (aromatic core with long alkyl chains) and hydrophilic (quarternized nitrogen centers) character in the molecule enhanced its propensity to aggregate in water. It is already known that this class of molecules form self-assembled physical gel in aqueous medium and also show propensity to salt-induced aggregation.[7] Considering their amenability to aggregation in water, we have been interested to explore their ability to detect heparin, a sulfated linear polymeric glycosaminoglycan in water.

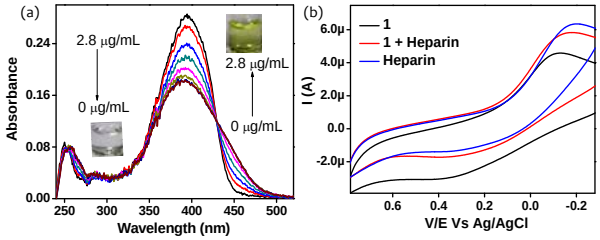

Fig. 1 (a) Change in the absorption spectra of **1** (10 μM) upon increasing concentration of heparin. (b) Cyclic voltammogram of **1** (0.1 mM) upon addition of heparin (0.1 mM) in water ([KCl] = 10 mM, scan rate = 200 mV/s).

Addition of heparin into an aqueous solution of **1** at pH 7.4 ([**1**] = 10 μM, [Heparin] = 2.5 μg/mL) resulted into vivid color change from nearly colorless to bright yellow (Fig 1a). UV-visible spectra on addition of heparin showed gradual appearence of the characteristic charge transfer band (ICT) at ~460 nm along with a concomitant hypsochromic shift in the absorption maximum (at 395 nm). This is due to the ground state charge transfer from negatively charged heparin to bis-pyridinium phenylenevinylene unit through effective aggregation in water. To ascertain the direction of charge transfer, cyclic voltamograms (CV) of **1** were recorded in presence of heparin in water at a scan rate of 200 mV.s^{-1} using glassy carbon as working electrode. In the cyclic voltammogram of the compound alone, a cathodic peak (reduction peak) appeared at -0.11 V (Ag/AgCl as reference electrode). Shift in reduction peak of **1** towards more negative potential value (-0.16 V) was observed upon addition of heparin. This indicated that the presence of negatively charged heparin moiety diminish the electron affinity of **1** (Fig 1b & S2, ESI†). This in turn suggests that the charge transfer occurrs from the electron rich heparin to the electron deficient bis-pyridinium moieties.

Interestingly, addition of heparin into the aqueous solution of **1** induced a unique concentration dependent emission switching sequentially from sky-blue, white, yellow and finally to orange. This remarkable alteration in the aggregation pattern could be easily evidenced from the diminution of the monomeric emission with simultaneous appearance of the red-shifted aggregated emission at ~600 nm (Fig 2a). The relative intensity ratios of these two bands eventually governed the color of the resultant emission. The CIE 1931 chromaticity diagram exhibits the coordinates for the corresponding changes in the emission colors and hence displays such emission switching (Fig. 2b). Cross-reactivity study in presence of potentially competing species is essential for real-life applications. To investigate the exclusive response of the probe towards heparin, several biologically relevant molecules were involved including chondroitin 4-sulfate (Chs) and hyaluronic acid (HA), the main interferents in the clinical evaluation of heparin. None of the other analytes except Chs and HA showed any detectable alterations in the emission spectrum of **1** (Fig. S3, ESI†). A slight enhancement in the intensity of the aggregated emission was observed upon addition of Chs and HA, which indicated that the electrostatic interaction along with the conformation of sugar dimer played a crucial role in the binding process. Higher negative charge density (4 negative charges per unit segment) of heparin might cause such preferential interaction with **1**. The affinity of **1** towards heparin was evaluated as 11.2 ± 0.01 (log K) based on 1:2 stoichiometry as predominently observed from Job's plot analysis. However, due to the complex polymeric nature, it may be difficult to predict a proper binding mode for the interaction of **1** with heparin, which was also evident from the variation in slope values at different regions of the Job plot curve (Fig. S4, ESI†).[8]

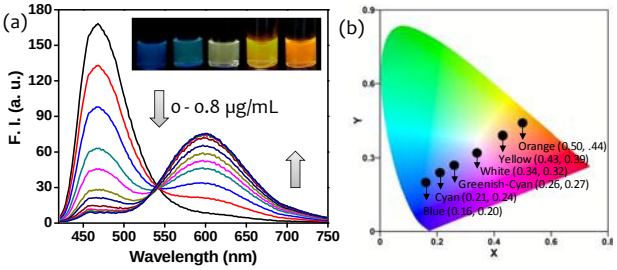

Fig. 2 (a) Changes in the emission spectra of **1** (10 μM, λ_{ex} = 400 nm) upon increasing concentration of heparin. [Changes in emission colors of **1** upon gradual addition of heparin (0-1.0 μg/mL) in water under a 365 nm UV lamp]. (b) CIE 1931 chromaticity diagram exhibiting the coordinates for the corresponding changes in emission colors.

A broad pH range 4.5-8.5 was found to be suitable for the recognition of analytes. At lower pH, the protonation at the carboxylate or sulphate ends of heparin might weaken the interaction, while at higher pH, the compound **1** was found to be in an aggregated state to begin with (Fig S5, ESI†). However, with increasing temperature the relative ratio of the aggregated emission and monomeric emission (I_{600nm}/I_{470nm}) decreased, which indicates certain extent of thermo-reversibility of the self-assembly process (Fig S6, ESI†). While the solution of **1** at low concentration was fairly stable in water, the heparin conjugate precipitated after 5 days due to electrical neutrality (Fig S7, ESI†). The heparin mediated self-assembly could be easily disassembled by adding requisite amount of protamine, a known heparin extractor through electrostatic interaction.[9] This enabled multiple time usability of the probe, which is an essential criterion of an ideal sensory system (Fig S8, ESI†).

Circular dichroism spectral analysis of the aggregates evidenced excellent chiral transcription upon addition of heparin. Heparin triggered self-assembly induced positive

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cotton effect and showed a peak first at 424 nm followed by a trough at 357 nm with a zero crossing at 378 nm, which indicated preferential formation of right handed helical structures (Fig 3a). To validate our speculation, atomic force microscope (AFM) images of **1** were examined in presence of heparin in water. Owing to a balance between hydrophobic/hydrophilic characters, the compound **1** alone showed moderate aggregation (spherical morphology with an average diameter of ~60-80 nm) in water. However, incorporation of heparin vividly changed the morphology of the mixture to fibrilar structures. Interestingly, the AFM images of heparin-conjugate showed large clump-like aggregates (size 180-250 nm) when kept for more than 5 days (Fig 3b).

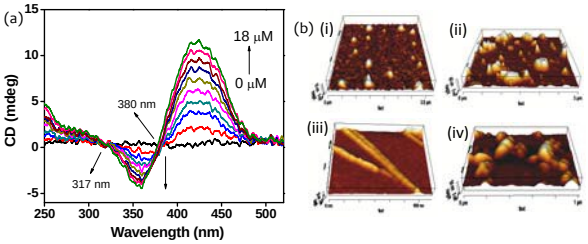

Fig. 3 (a) Changes in the circular dichroism spectra of **1** (10 μM) upon addition in increasing concentration of heparin (pH 7.4). (b) AFM images of (i) **1** (10 μM), (ii) heparin alone (1.0 μg/mL, (iii) **1** in presence of heparin (1.0 μg/mL) and (iv) heparin conjugate after 5 days.

Time dependent emission spectra (TCSPC) of **1** showed mono-exponential decay with a time constant of 0.13 ns (χ^2=1.10) when monitored at 470 nm in water (Fig 4a). However, addition of heparin in this condition induced multi-exponential decays (λ_{em}=600 nm) with relatively longer average lifetime of 9.4 ns (χ^2=1.15) (Table S1, ESI†). This essentially indicates heparin mediated aggregation of the probe in water (pH 7.4).[10] ^{1}H-NMR spectra of compound **1** in D_2O upon addition of heparin exhibited broadening of signals (almost quenching). This broadening and subsequent disappearance of the proton signals unambiguously establishes the effect of strong intermolecular interactions leading to the aggregate formation (Fig S9, ESI†).[11] Thus, from the above evidences one may conclude that the compound **1**, having positively charged pyridinium ends remains mostly in monomeric form in water. However, in presence of heparin (a negatively charged glycopolymer), it can form electrostatic complex, leading to the formation of fibrilar aggregate.

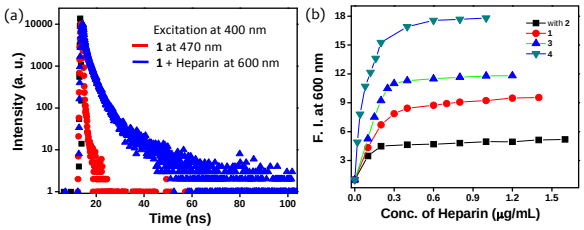

Fig. 4 (a) Emission decay profile of **1** (10 μM, λ_{ex} = 400 nm) monitored at both 470 and 600 nm in water (pH 7.4). (b) Change in emission intensity of different compounds (10 μM, λ_{ex} = 400 nm) at 600 nm upon addition of heparin.

To elucidate the importance of the length of the alkyl chain in the self-assembly process, we studied three other probe compounds with different alkyl chains (-C_6H_{13}, -$C_{10}H_{21}$ and -$C_{12}H_{25}$). In each case the mode of interaction with heparin was found to be almost similar (as evident from spectral changes) (Table S2, ESI†). However, the extent of interaction was found to be higher for compounds having longer alkyl chains. This indicated that the presence of longer alkyl chain rendered the aggregation with heparin more facile (Fig 4b & S10, ESI†). Though the compound with longer alkyl chains showed larger propensity to aggregation with heparin, the compound **1** having *n*-octyl chains was considered for further practical applications because the solutions of the compounds with longer alkyl chains were relatively less stable in water and precipitated after 12-14 h. To ascertain the importance of the core aromatic phenylenevinylene unit, we have introduced a new viologen compound **6** having in -C_8H_{17}-alkyl chains at both the pyridinium ends. As expected, this compound showed less effective interaction with heparin in water due to the lack of avaliability of planar aromatic core in it for easy aggregation (Fig S11, ESI†). On the other hand, the control compound **5** without the positive terminal ends also did not evidence any interaction with heparin (Fig S12, ESI†).

Considering the excellent selectivity of the probe **1** towards heparin in water, we checked the sensitivity of the probe in fetal bovine serum (FBS) medium. Progressive addition of heparin in diluted FBS (5%) medium resulted in a gradual increase in the aggregated emission in expense of that of the monomeric one. However, the saturation concentration of heparin in serum was found to be higher (~3 fold) compared to normal buffered solution (pH 7.4) (Fig S13b, ESI†). This might be due to the association of the probe molecules inside the hydrophobic cavity of the serum albumins. The lowest detectable concentration of heparin in this case was estimated to be ~12.5 ng/mL, which satisfies the requirement of clinical heparin monitoring during cardiovascular surgeries (Fig S14, ESI†). Most importantly, the presence of other ionic components of serum at physiological level did not show any significant interactions with **1** (Fig S13a, ESI†).

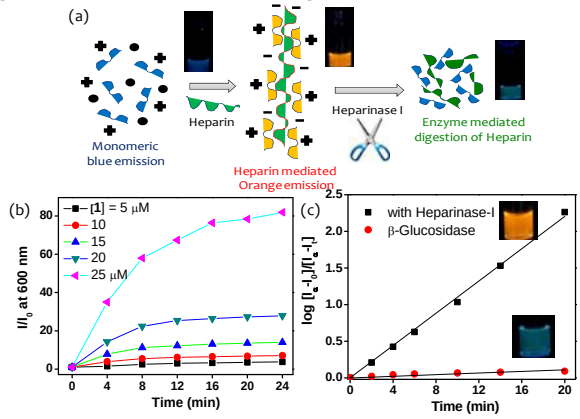

Fig. 5 (a) Schematic diagram showing Heparinase I mediated 'breaking up' of the **1**+heparin conjugate in water. (b) Effect of probe **1** concentration on interaction of heparinase I (0.2 μg/mL) at pH 7.4. (c) Comparison of the interaction of heparinase I and β-glucosidase with **1**+heparin conjugate in water (pH 7.4).

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The level of glycosaminoglycans (GAGs) modifying enzymes in human plasma is an important bio-marker of the progression of many lethal diseases including cancer. For example, an enhancement in heparinase activity is considered to be an indication of multiple myeloma.[12] However, fluorometric assay for heparinase is really scarce in litereture.[13] Therefore, we were interested to employ the heparin mediated reversible aggregation of our probe molecules for fluorometric estimation of heparinase I. A drastic quenching in the aggregate emission (at 600 nm) was observed upon incubation of the pre-formed **1**+heparin conjugate with heparinase I for ~30 min (Fig S15, ESI†). The degradation process was observed to follow a 1st order kinetics in the presence of excess of enzyme (Fig S16a, ESI†). The Michalis-Menten constant was determined to be 2.32 x 10^{-6} M for heparinase I following the present protocol, which appeared to be in good agreement with the literature (Fig 5b & S16b, ESI†).[14] To ensure the specificity of this newly developed protocol the enzymatic assay was also performed in the presence of normal β-glucosidase. However, in this case no spectral change was identified even after 20 min of incubation at pH 7.4 (Fig 5c). Further heparinase estimation was also achieved in diluted serum (5%) medium. Here also a decrease in aggregated emission was observed upon incubation of **1**+heparin conjugate with enzyme for requisite time (S17, ESI†).

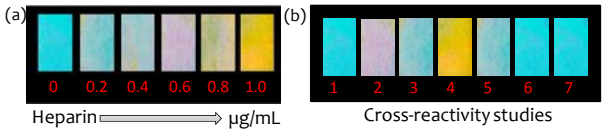

Fig. 6 (a) Color changes of compound coated TLC plates upon dipping into the solution of heparin of different concentration in water (pH 7.4) [under ~360 nm UV light]. (b) Color change (under ~360 nm UV lamp) of compound coated TLC plates upon dipping into different analyte solution; No analyte [**1**] and with Chs [**2**], HA [**3**], Heparin [**4**], Protamine [**5**], Polylysine [**6**], Chitosan [**7**] (pH 7.4).

Further, the dose-dependent changes in emission signal with heparin encouraged us to develop easily obtainable solid-support based portable devices for on-site heparin detection and quantification.[15] A gradual change in color from sky-blue to yellow (under long UV light) was observed upon dipping the compound coated TLC plates into heparin solution of different concentrations (Fig 6a). Control experiments were also designed by dipping the strips into solution of HA and Chs to verify the selectivity of the present protocol (Fig 6b). No detectable color change in these two cases ensured the specificity of the sensor towards heparin over the other potential competitors. Thus, it could be easily concluded that the present protocol assured the rapid on-site identification of heparin along with proper quantification.

Conclusions

In conclusion, we have introduced conjugated bis-pyridinium phenylenevinylene based molecular probes for their unique heparin triggered dose-dependent, multi-color emission switching at physiological pH in water. The heparin induced reversible aggregate formation was further utilized in developing a new easy-to-operate assay for heparinase I in biological fluids. The dose dependent emission modulation was also observed on low-cost color strips, which ensured rapid onsite detection of heparin in unknown samples.

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