

A spectroscopic study of interaction of cationic dyes with heparin

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Received: 03 September 2009; revised: 09 November 2009; accepted: 22 November 2009. Available online: 09 January 2010.

ABSTRACT: The interaction of two cationic dyes namely, acridine orange and pinacyanol chloride with an anionic polyelectrolyte, heparin, has been investigated by spectrophotometric method. The polymer induced metachromasy in the dyes resulting in the shift of the absorption maxima of the dyes towards shorter wavelengths. The stability of the complexes formed between acridine orange and heparin was found to be lesser than that formed between pinacyanol chloride and heparin. This fact was further confirmed by reversal studies using alcohols, urea and surfactants. The interaction of acridine orange with heparin has also been investigated fluorimetrically. The interaction parameters revealed that binding between acridine orange and heparin arises due to electrostatic interaction while that between pinacyanol chloride and heparin is found to involve both electrostatic and hydrophobic forces. The effect of the structure of the dye in inducing metachromasy has also been discussed.

Keywords: cationic dyes, metachromatic complex, heparin, fluorescence quenching, structure, aggregation

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Introduction

Metachromasy is a well-known phenomenon in the case of dye-polymer interactions and is generally found in the case of aggregation of cationic dyes on anionic polymers [1]. The metachromatic change, which is most pronounced in the visible spectrum, is frequently characterized by the appearance of a new absorption band. The change has been attributed due to the formation of various dye aggregates of the bound dye molecules on the polymer sites [2]. A similar change occurs with the cationic dyes in aqueous solution, as their concentration is increased and the temperature is decreased. The metachromatic changes are frequently specific rather than general and constitute an experimental basis of histo and cytochemical applications. For a particular polymer species it often depends on the specific substituent attached to the dyes even if they belong to a class of same chemical structure [3, 4]. The interaction of polyphosphates and cationic dyes such as toluidine blue [5], 4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiocarbocyanine [6], acridine orange and proflavine [7-11] are reported. The interaction of heparin with methylene blue and Azure A [12-16] are already reported in the literature. The phenomena of reversal of metachromasy by addition of urea, alcohols, neutral electrolytes [17] and also by increasing the temperature of the system, may be used to determine the stability of the metachromatic compound.

The fluorescence of the dye acridine orange (**1**) and its quenching in polymer matrices has been studied extensively [18-20]. Hence the objective of the present study is to understand the forces involved in the binding of the dyes to the anionic polyelectrolyte and also to evaluate the thermodynamic parameters of interaction. Moreover, pinacyanol chloride (**2**) is larger in size and would yield better information on dye polymer interaction. Hence it has been selected for our present studies. The equivalent weight of heparin was found to be 178 [21-24].

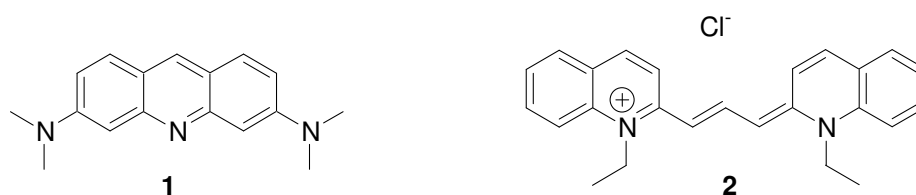


Figure 1. Structures of acridine orange (**1**) and pinacyanol chloride (**2**).

Material and Methods

Reagents

Acridine orange (AO), was obtained from S. D. Fine Chemicals, Mumbai and used as received. Pinacyanol chloride (Pcyn) was obtained from Acros media and used as received. Sodium heparinate (NaHep) (Lobachemie, India) was used as received; Methanol, ethanol and 2-Propanol (Merck, India) were distilled before use. Urea, sodium chloride, potassium chloride, sodium lauryl sulphate and sodium dodecyl benzene sulphonate (Loba Chemie, India) were used as received. Absorption Spectra were recorded using a Shimadzu UV-2550 Spectrophotometer. The concentrations of the stock solution of the dye and the polymer were 6×10^{-5} M and 1×10^{-3} M, respectively.

Determination of stoichiometry of polymer-dye complex

Increasing amounts of polymer solution (0.0-9ml, 1×10^{-3} M) were added to a fixed volume of dye solution (0.6mL, 1×10^{-3} M) in case of acridine orange and (0.5mL, 1×10^{-3} M) in different sets of experiments and the total volume was made up to 10 mL by adding distilled water in each case. The absorbances were measured at the respective monomeric and metachromatic bands.

Effect of alcohols and urea on the absorption of pure dye

The effect of alcohols namely, methanol, ethanol, 2-propanol and urea on the spectra of the pure dye was studied by measuring absorbance of the pure dye solution at 492 nm in case of acridine orange and at 600 nm in case of pinacyanol chloride in the presence of alcohols and urea. 10 mL solutions were made with 0.6 mL of 1×10^{-3} M dye and remaining alcohol (10-80%) or aqueous urea (1-8 M) and the absorbances were recorded at the respective monomeric band.

Study of reversal of metachromasy using polymer-dye complex

For measurements of the reversal of metachromasy, solutions containing polymer and dye in the ratio 2.0:1.0 were made containing different amount of alcohol. The total volume was maintained at 10 mL in each case. The absorbances were measured at 492 nm and 457 nm in case of acridine orange and at 600 nm and 486 nm in case of pinacyanol chloride. Similarly, polymer-dye solutions containing different amounts of urea (1-8 M) were made and the absorbances were measured at 492 nm and 457 nm in case of acridine orange-Heparin complex and at 600 nm and at 486 nm in case of Pinacyanol chloride-Heparin complex.

Fluorescent studies

Fluorescence of the dye solutions as well as that of the dye-polymer mixture was measured using Jasco FP-6200 Spectrofluorometer. Spectrofluorometric titrations were carried out by measuring fluorescence intensity of the AO solutions, at the emission peak, 529 nm upon addition of increasing amount of polymer sample under separate experiments. The concentration of the aqueous solution of the dye was 6×10^{-5} M. The

concentration of the polymer solution varied from 10^{-3} M- 10^{-4} M. Increasing amounts of polymer solution were (0.0mL-9 mL, 1×10^{-3} M) added to fixed volume of dye solution in different sets of experiments and the total volume was made upto 10 mL in different sets of experiments and the total volume was made up to 10 mL by adding distilled water in each case.

Determination of thermodynamic parameters

The thermodynamic parameters were determined by measuring the absorbances of the pure dye solution at both the respective monomeric and metachromatic band in the temperature range (36-54 °C). The above experiments were repeated in presence of the polymer at various polymer-dye ratios (2, 5, 10, and 20).

Determination of equivalent weight of heparin

The given sample of sodium heparinate was completely converted to heparinic acid. To ensure complete conversion of sodium heparinate to heparinic acid, each sample was passed through a column of approximately 50-fold excess Dowex-50W (H^+ , form, x 4, 200 – 400 mesh) immediately before titration. The possibility of hold up of heparin on the column was eliminated by subsequent passage of ten-fold excess, over heparin, of 4 N [21-23]. The ratio of number of sulphate to carboxylate groups in heparin was determined by conductometric titration method [24]. The equivalent weight of heparin was found to be 178.

Results and Discussion

The absorption spectra of acridine orange (**1**) and pinacyanol chloride (**2**) at various concentrations are shown in figures 2 and 3, respectively. The maxima absorption was found to be 492 nm in case of acridine orange and 600 nm in case of pinacyanol chloride, indicating the presence of a monomeric dye species in the concentration range studied. On adding increasing amounts of polymer solution the absorption maxima shifts to 457 nm in case of AO-Heparin and at 486 nm in case of pinacyanol chloride-heparin complex. The blue shifted band is attributed to the stacking of the dye molecules on the polymer backbone and this reflects high degree of cooperativity in binding [25]. Appearance of multiple banded spectra proposed that the polymer might have a random coil structure in solution. Whereas at higher concentration of the polymer almost a single banded spectrum was observed due to possible change from random coil to helical form. In this regard the accepted mechanism suggests that the dye cations bind to adjacent sites of the polymer forming a single individual compound, which is considered to arise from electrostatic interaction among neighboring

dye molecules and fixed sites on polymer as a result of which they suffer effective aggregation resulting in hypochromic and hypsochromic spectral shifts in the absorption band of the dye [26]. The results are shown in figures 4 and 5, respectively.

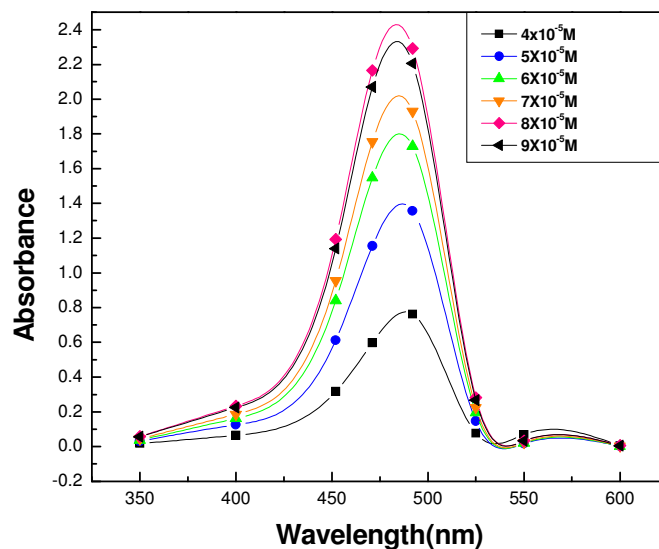


Figure 2. Absorption spectrum of dye, acridine orange at various concentrations.

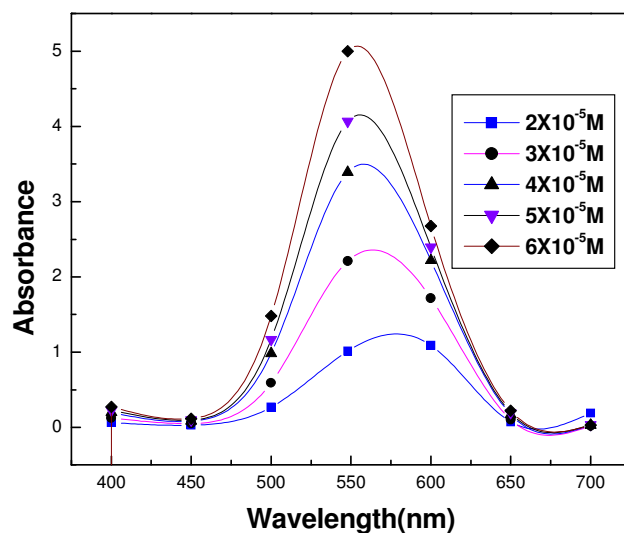


Figure 3. Absorption spectrum of dye, pinacyanol chloride at various concentrations.

Determination of stoichiometry

To determine the stoichiometry of the polymer-dye complex, a plot of A_{457}/A_{492} versus the polymer/dye ratio was made. The stoichiometry of AO-NaHep complex was found 1:1.5 which indicates that the binding is at adjacent anionic sites. This

stoichiometry indicates that every potential anionic site of the polyanion was associated with the dye cation and aggregation of such dye molecules was expected to lead to the formation of a card pack stacking of the individual monomers on the surface of the polyanion so that the allowed transition produces a blue-shifted metachromasy [27]. While in case of pinacyanol chloride-heparin complex the stoichiometry is 1:2 and the binding is at alternate anionic sites. This indicates that there is lesser overcrowding and more aggregation of the bound dyes on the polymer chain in the latter case than in the former case. Similar results were reported in case of binding of pinacyanol chloride on poly(methacrylic acid) and poly(styrene sulfonate) systems [28]. The results are shown in figures 6 and 7, respectively.

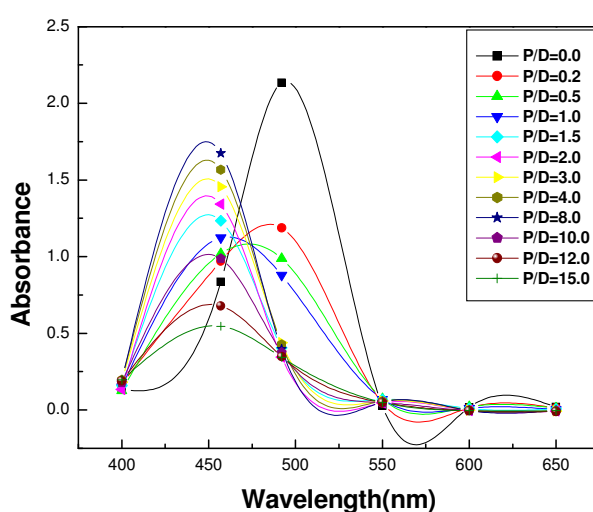


Figure 4. Absorption spectrum of AO-NaHep system at various P/D ratios.

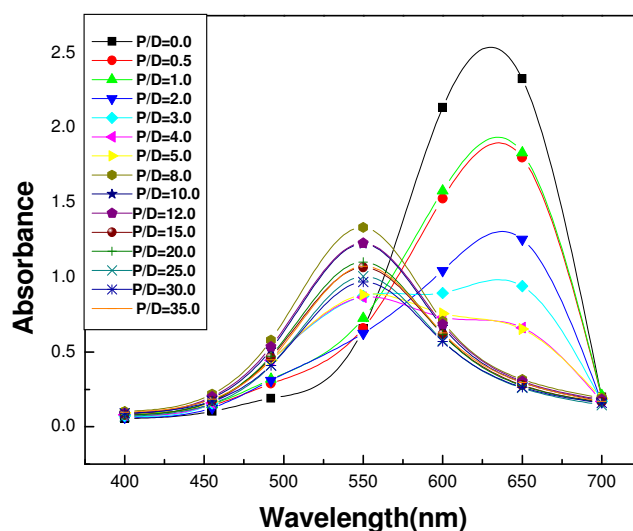


Figure 5. Absorption spectrum of pinacyanol chloride-NaHep system at various P/D ratios.

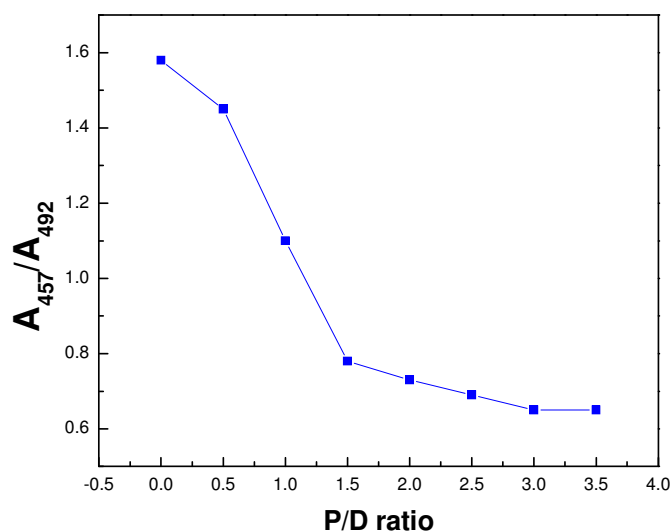


Figure 6. Stoichiometry of AO-NaHep complex.

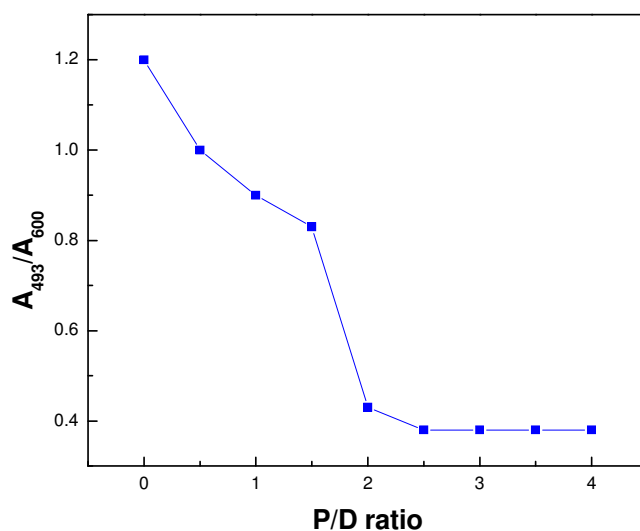


Figure 7. Stoichiometry of Pcyn-NaHep complex.

Reversal of metachromasy using alcohols and urea:

The metachromatic effect is presumably due to the association of the dye molecules on binding with the polyanion which may involve both electrostatic and hydrophobic interactions. The destruction of metachromatic effect may occur on addition of low molecular weight electrolytes, alcohols or urea. The destruction of metachromasy by alcohol and urea is attributed to the involvement of hydrophobic bonding has already been established [29-32]. The efficiency of alcohols in disrupting metachromasy was

found to be in the order methanol<ethanol<2-propanol, indicating that reversal becomes quicker with increasing hydrophobic character of the alcohols. The above facts are further confirmed established in the present system. On addition of increasing amount of alcohol to the polymer/dye system at P/D=2.0, the original monomeric band of dye species is gradually restored. The efficiency of the alcohols, namely methanol, ethanol and propanol, on destruction of metachromasy was studied as shown in figures 8 and 9, 50% methanol, 40% ethanol, 30% 2-propanol were required to reverse metachromasy in P-heparin system. While in case of AO-Heparin system 50% methanol, 25% ethanol, 20% 2-propanol were sufficient to reverse metachromasy. However, the concentration of urea to reverse metachromasy is found to be as high as 5 M in P-Hep system and 4 M in case of AO-Hep system (figures 10 and 11). Similar reports are available in literature for reversal of metachromasy in anionic polyelectrolyte/cationic systems by addition of alcohols or urea [33-37].

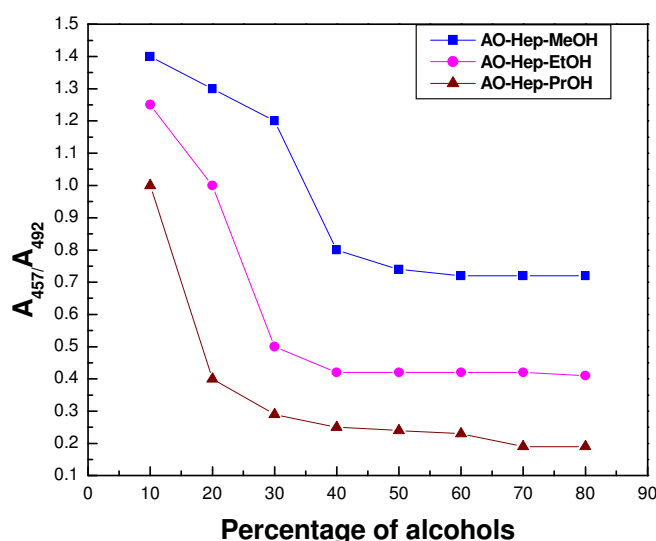


Figure 8. Reversal of metachromasy on addition of alcohols in AO-Hep systems.

Effect of surfactants

The strength and nature of interaction between water soluble polyelectrolyte and oppositely charged surfactants depend on the characteristic features of both the polyelectrolyte and the surfactant. The charge density, flexibility of the polyelectrolyte and the hydrophobicity of the non-polar part and the bulkiness of the polar part also play a vital role in the case of polysaccharide-surfactant interaction [38]. On adding increasing amounts of sodium laurylsulphate and sodium dodecylbenzene sulphonate to AO-sodium heparin complex the molar concentrations of sodium laurylsulphate and sodium dodecylbenzene sulphonate needed to cause reversal was found to be 1×10^{-3} M and $1 \times 10^{-}$

⁴ Min case of AO-Hep and 1×10^{-3} M and 1×10^{-2} M in case of P-Hep system. These results agree with those reported earlier in literature [39]. Thus the addition of surfactants causes the production of micelles. Thus the surfactant molecules interacted with the polymer by replacing the cationic dye. The release of dye molecules from the dye-polymer complex in presence of cationic surfactants revealed that surfactants interacted electrostatically [40] with the anionic site of the polymer and thus the dye becomes free. The ease of reversal of metachromasy can be correlated with its chain length [41]. Thus the binding between oppositely charged polymer surfactant is primarily electrostatic force which is reinforced by hydrophobic forces. The results are shown in figures 12 and 13, respectively.

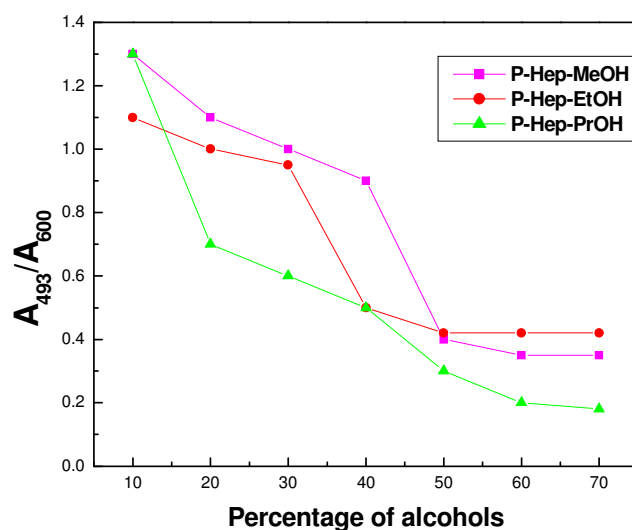


Figure 9. Reversal of metachromasy on addition of alcohols in Pcyn-Hep systems.

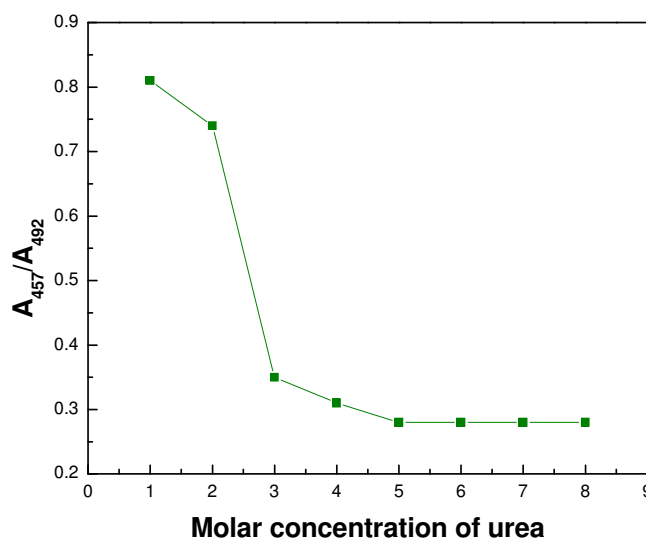


Figure 10. Reversal of metachromasy on addition of urea in AO-Hep complex.

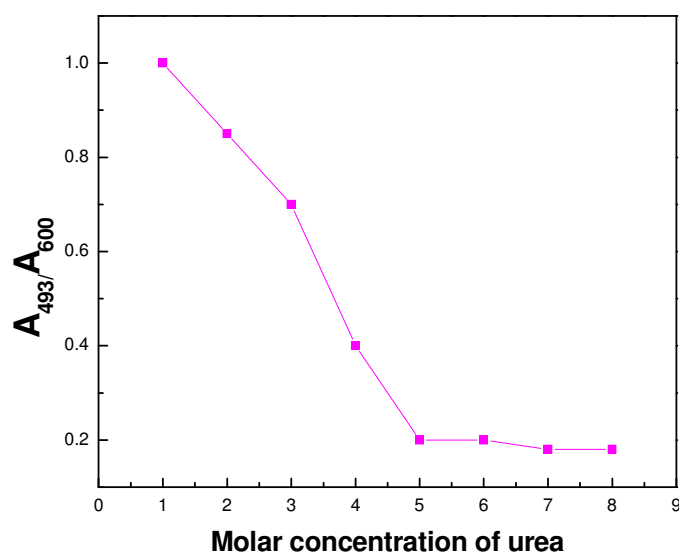


Figure 11. Reversal of metachromasy on addition of urea in Pcyn-Hep complex.

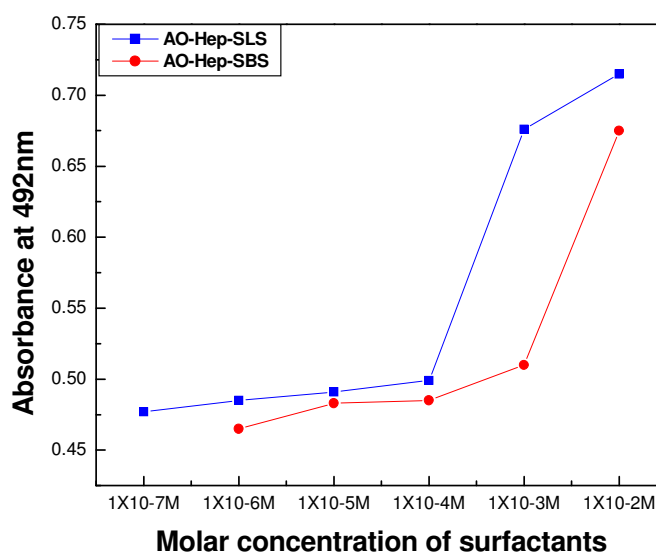


Figure 12. Reversal of metachromasy on addition of surfactants in AO-Hep complex.

Effect of ionic strength:

Tan & Schneider [42] have reported the disruption of metachromatic band with the variation of ionic strength. When sodium chloride of varied ionic strength were added to AO-Hep and Pcyn-Hep complex and the absorbances were measured in the range 350-700 nm. In case of Pcyn-Hep the monomeric band reappears at higher ionic strength (0.1 M) than compared to that of AO-Hep (1×10^{-3} M). In aqueous solutions the charged polymer molecule will be in the extended conformation due to the repulsion between the charged groups. On adding the dye the conformation of the polycation, changes to a

compact coil owing to dye binding, resulting from electrostatic interaction thus giving rise to metachromatic band. The concentration of sodium chloride required to reverse metachromasy was greater in case of pincyanolchloride-heparin complex than in the case of acridine orange-heparin complex. The results are shown in figures 14 and 15, respectively.

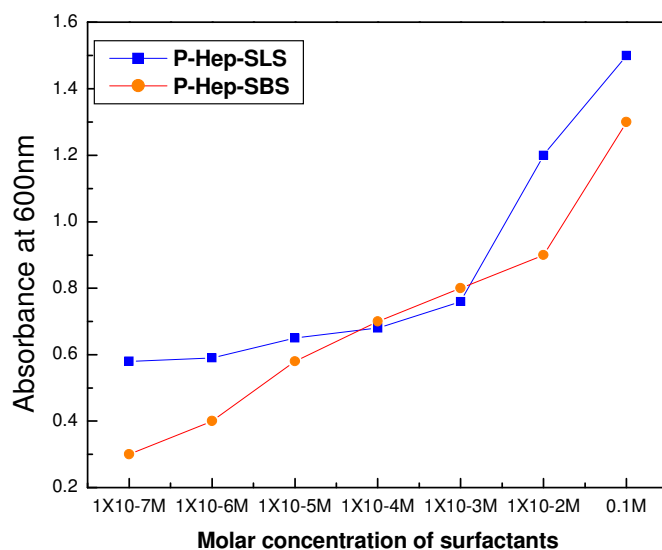


Figure 13. Reversal of metachromasy on addition of surfactants in P-cyn-Hep complex.

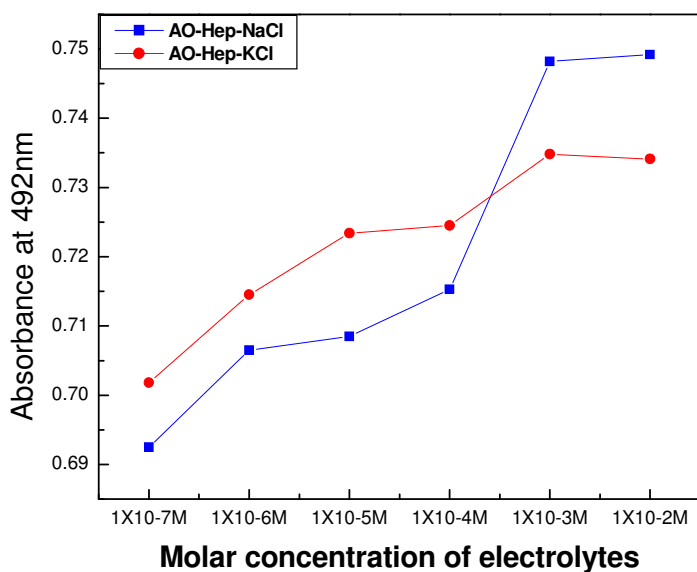


Figure 14. Reversal of metachromasy on addition of electrolytes in AO-NaHep complex.

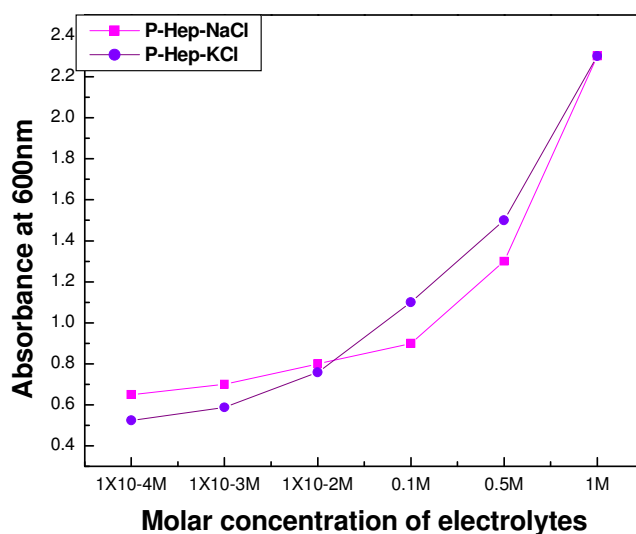


Figure 15. Reversal of metachromasy on addition of electrolytes in Pcyn-NaHep complex.

Determination of interaction parameters

The interaction constant K_c for the complex formation between AO and NaHep and Pcyn-NaHep was determined by absorbance measurements at the metachromatic bands at four different temperatures taking different sets of solutions containing varying amounts of polymer (C_s) in a fixed volume of the dye solution. The value of K_c was obtained from the slope and intercept of the plot of $C_D C_S / (A - A_0)$ against C_s shown in figures 16 and 17. Absorbance results were treated using Rose-Drago eqn. [43], $C_D C_S / (A - A_0) = 1/K_c L (\epsilon_{ds} - \epsilon_d) + C_S / L (\epsilon_{ds} - \epsilon_d)$. C_D refers to the initial molar concentration of dye and C_S refers to the concentration of the polymer sample. In each case the thermodynamic parameters of interaction, namely ΔH , ΔG and ΔS were also calculated (figures 18 and 19). The results are given in the Table 1.

- Calculated from figures 16 and 17 according to Rose-Drago equation.
- Calculated from the thermodynamic equation $\Delta G = -RT \ln K_c$.
- Calculated graphically by plotting $\ln K_c$ against $1/T$ according to Van't Hoff equation,

$$\ln K_c = -\Delta H / RT + C.$$

- Calculated from the thermodynamic expression $\Delta G = \Delta H - T\Delta S$.

The values of interaction constant of AO-NaHep and Pcyn-NaHep decreases with rise in temperature suggesting the exothermic nature of the reactions and such a low

value ΔG suggested a non-chemical type of interaction. The negative value of entropy indicated a more ordered state of the ions due to its aggregation. Thus all these thermodynamic parameters suggested the interaction between the anionic sites of the polyanions and the counter ions resulting in aggregation and induction of metachromasy.

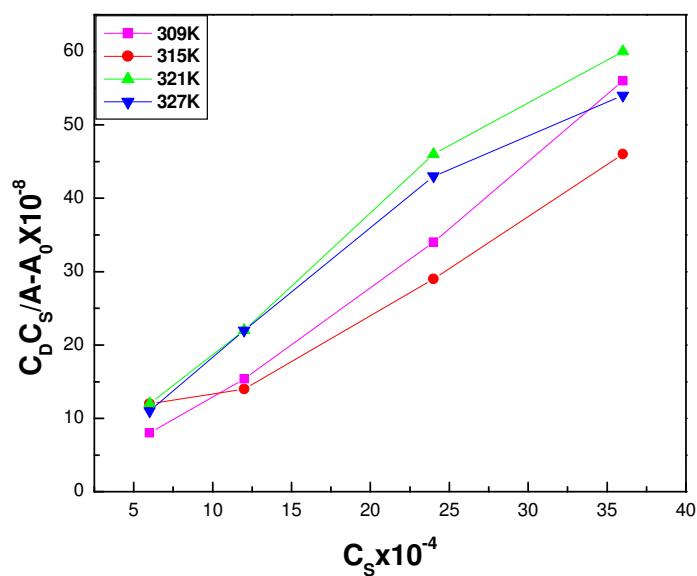


Figure 16. Plots of $C_D.C_S/(A-A_0)$ against C_S for AO-NaHep system at different temperatures.

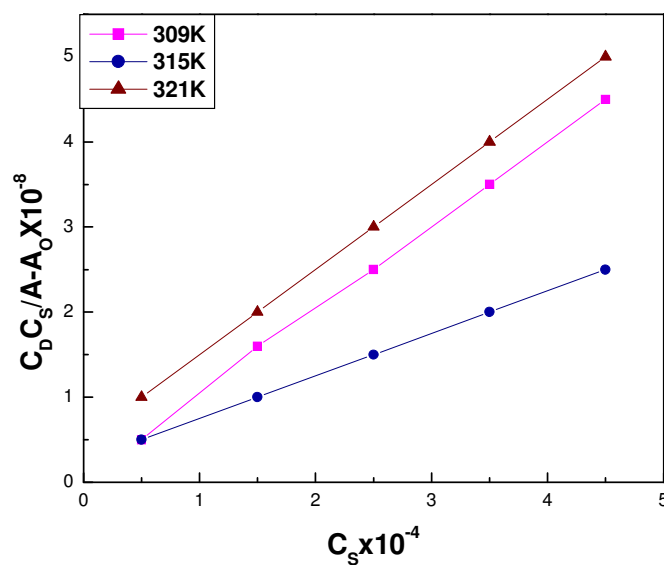


Figure 17. Plots of $C_D.C_S/(A-A_0)$ against C_S for Pcyn-NaHep system at different temperatures.

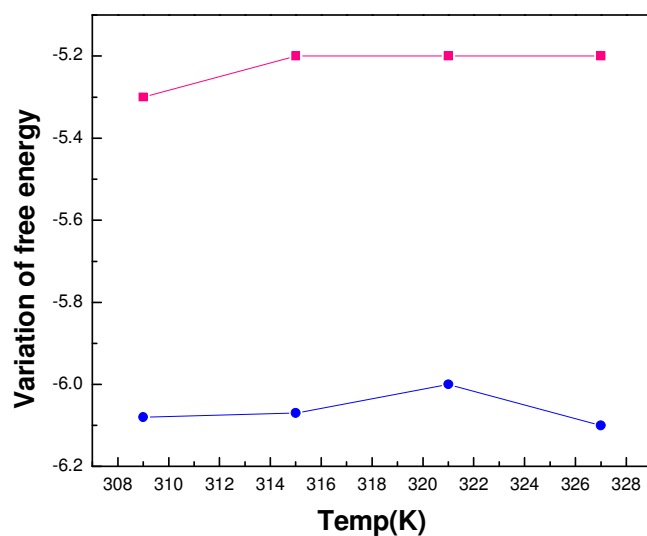


Figure 18. Variation of ΔG with temperature in AO-NaHep and Pcyn-NaHep systems.

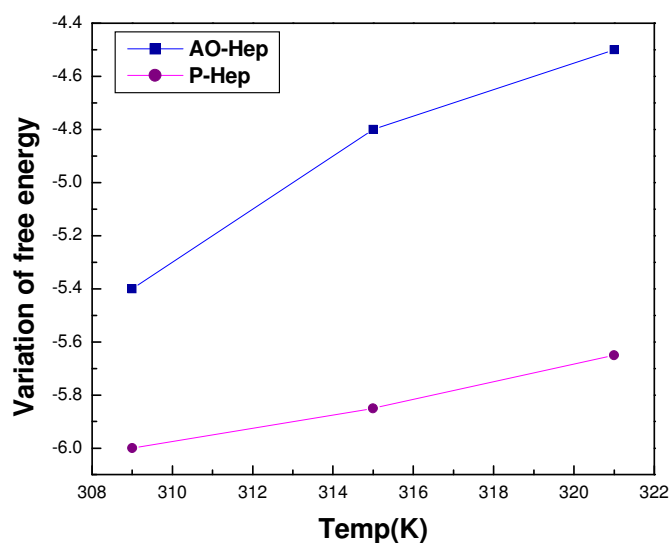


Figure 19. Van't Hoff plots for AO-NaHep and Pcyn-NaHep systems.

Fluorescence measurements

Fluorescent studies were performed with AO-NaHep system and it was found that the fluorescent intensity of AO decreased on the addition of increasing amounts [43] of polymer solution as evidenced from Figure 20. This indicated that the planar cationic dye AO preferred to form aggregates of dye molecules on binding to heparin. Such fluorescence quenching of AO by various bacterial polysaccharides are already reported

in the literature [44]. Finally, to study the interaction between the polymer and dye, the fluorescence data were fitted to Stern-Volmer equation, $F_0/F=1+K_{sv}[Q]$, where F_0 is the fluorescence intensity of the dye solution and F is that of the dye-polymer mixture, and $[Q]$ indicated the molar concentration of the polymer, K_{sv} is the Stern-Volmer constant. The Stern-Volmer plots obtained for the present data is shown in Figure 21. From the slope of the plot of F_0/F the value of the Stern-Volmer plot found to be $1.1 \times 10^3 \text{ lit}^{-1} \text{ mol}^{-1}$, which is less than that reported for the interaction between AO-Klesiella. One possible explanation for that may be due to the self-association of quencher molecule, or the quencher with the fluorescer as reported in the literature. The linearity of the plot indicated that the quenching in this case is static in nature [45].

Table 1. Thermodynamic Parameters for interaction of AO-NaHep and Pcyn-NaHep

Temp (K)	KC(dm ³ mol ⁻¹) ^a		$\Delta G(\text{kcal. mol}^{-1})^b$		$\Delta H (\text{kcal. mol}^{-1})^c$		$\Delta S(\text{cal. mol}^{-1} \text{K}^{-1})^d$	
	AO-Hep	P-Hep	AO-Hep	P-Hep	AO-Hep	P-Hep	AO-Hep	P-Hep
309	5400	20000	-5.3	-6.08				
315	3500	16666	-5.29	-6.07	-6.724	-7.316	- .0046	-.004
321	1600	13820	-5.25	-6.0				
327	1062	10020	-5.22	-6.1				

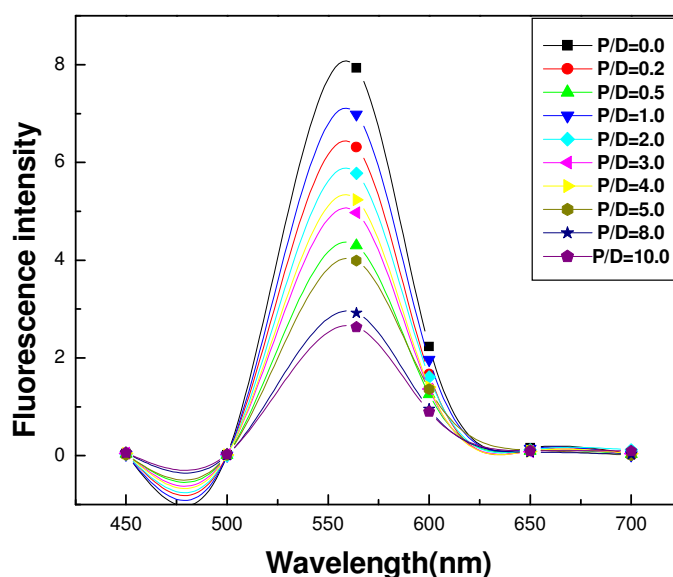


Figure 20. Emission spectra of AO -NaHep system at various P/D ratios.

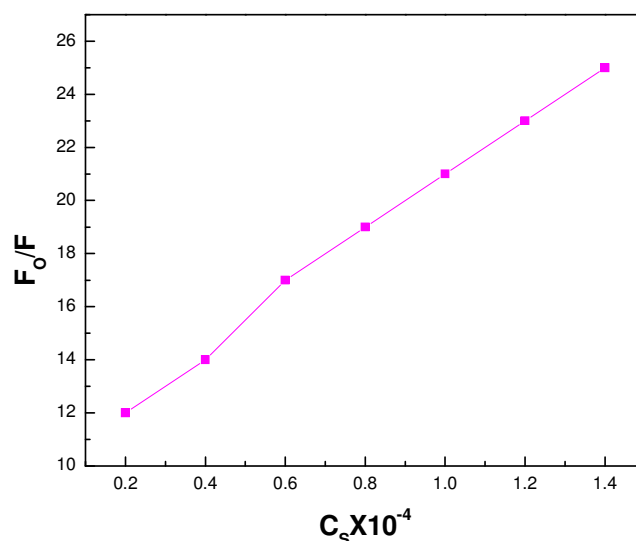


Figure 21. Stern-Volmer plot for AO-NaHep complex.

Effect structure of the dye

The structures of acridine orange and pinacyanol chloride are given at Figure 1. Thus it is evident that acridine orange, being a rigid planar cationic dye and hence a small distance between the adjacent anionic sites on the polyanion will be more favorable for binding resulting in stacking arrangement. On the other hand, pinacyanol chloride being larger in size is more hydrophobic and hence induces greater aggregation. Thus in this case the distance between the two adjacent dye molecules will be greater and the dye molecules are oriented like a stair case which agrees with the reported literature [46]. Furthermore the dye molecules are arranged in a parallel and stacked manner within the aggregates resulting in a hypsochromic shift [47].

Conclusion

The polymer heparin is observed to be effective in inducing metachromasy in the dyes AO and pinacyanol chloride. The metachromatic spectral shift is 35 nm in case of AO-Heparin and 114 nm in case of P-Heparin this can be correlated to the structure of the dye. Thus pinacyanol chloride, a cyanine dye is more hydrophobic and more aggregating in nature. The 2:1 stoichiometry indicates binding is at alternate anionic sites in case of P-Hep whereas in case of AO-Heparin the binding is at adjacent anionic sites. The study of the effect of alcohols and urea on the reversal of metachromasy indicates weak electrostatic binding between AO-NaHep, which is evident from the

thermodynamic data and fluorescence study. Thus the binding between Pcyn-NaHep is governed by strong electrostatic interaction reinforced by hydrophobic interaction. Thus the spectral studies prove the chromotropic character of NaHep and the cooperative nature of the dye-polymer interactions in various systems.

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